

Für Moritz

Towards a second generation of *Salmonella*-mediated oral DNA vaccines

Von der Gemeinsamen Naturwissenschaftlichen Fakultät
der Technischen Universität Carolo-Wilhelmina
zu Braunschweig
zur Erlangung des Grades einer
Doktorin der Naturwissenschaften
(Dr.rer.nat.)
genehmigte
D i s s e r t a t i o n

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eingereicht am: 22.12.2003

mündliche Prüfung (Disputation) am: 13.04.2004

Vorveröffentlichungen der Dissertation

Teilergebnisse aus dieser Arbeit wurden mit Genehmigung der Gemeinsamen Naturwissenschaftlichen Fakultät, vertreten durch den Mentor der Arbeit, in folgenden Beiträgen vorab veröffentlicht:

Tagungsbeiträge

Bauer, H.; Darji, A.; Chakraborty, T. and Weiss, S.: Improvement of eukaryotic expression plasmids for oral genetic vaccination using attenuated *Salmonella* as carrier (Poster) 34th Annual Meeting of the German Society of Immunology, Berlin (2003)

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1 Introduction

1.1 Vaccination

The most efficacious and cost-effective possibility to interfere with infectious diseases is the prophylactic immunization against pathogens. Therefore, the development of new or improved vaccines is one of the most important challenges in modern medicine to prevent human morbidity and mortality caused by different infectious microorganisms.

The early concept of vaccination is based on the observation by Edward Jenner in 1796 that prior exposure to cowpox (vaccinia) could protect against subsequent challenge with virulent smallpox (Jenner, 1798). Pasteur, who developed over hundred years later a vaccine against chicken cholera (Pasteur, 1880), extended the term vaccination to the stimulation of protection to other infectious agents (Silverstein, 1998).

The development and widespread use of vaccines against a variety of pathogens has been a great triumph of medical science, and many major diseases, such as diphtheria, poliomyelitis and measles are kept under control in industrialized countries. In the case of smallpox, it even resulted in a worldwide eradication of the pathogen.

Despite this outstanding success, vaccines for many diseases throughout the world, like AIDS caused by the human immunodeficiency virus (HIV), tuberculosis caused by *Mycobacterium tuberculosis*, leishmaniasis caused by *Leishmania major* and malaria caused by *Plasmodium falciparum* are either not available or ineffective. Therefore, there is a growing need for the development of completely new vaccines, besides improvement of existing vaccines in terms of efficacy and safety.

1.1.1 Requirements for an effective vaccine

According to the nature of the infecting organism, the particular requirements for successful vaccination can vary extremely. Whereas humoral responses, mediated by antibodies, can directly attack microorganisms and subsequently prevent infection, T cells as instruments of cellular immune responses work by killing infected cells. Thus, T cells cannot prevent infection; rather they contribute to host defense and clearance of pathogens (Srivastava and Liu, 2003). The ideal vaccine therefore provides host defense already at the point of entry of the pathogen. Consequently, the stimulation of mucosal immunity plays an important role in vaccination, since most infectious organisms enter the host through mucosal surfaces.

Immunity against most viral and bacterial infections is mediated by a humoral immune response (production of antibodies). Some microorganisms such as the causative agents of tetanus and diphtheria, *Clostridium tetani* and *Corynebacterium diphtheria*, respectively, as well as the intracellular poliomyelitis virus require the presence of preexisting neutralizing antibodies for an effective protective immunity (Makela, 2000). For intracellular infectious microorganisms such as *Mycobacterium tuberculosis*, *Leishmania major*, and other parasites, protection is mediated by cellular immunity. Moreover, for some diseases (e.g. HIV infection, herpes and malaria), both humoral and cellular responses are likely to be required. The cellular immune responses comprise primarily CD4⁺ and CD8⁺ T cells in these cases (Gurunathan et al., 2000). This demonstrates the versatility of problems that need to be tackled in vaccine design.

In addition, one very important criterion for a successful vaccine is its ability to produce protective immunity against exposure to live pathogens in a very high proportion of the population. Such a vaccine should generate long-lived immunological B as well as T cell memory to give sustained protection already after a single application, as it is impractical to administer booster vaccinations to large or dispersed rural populations. Furthermore, the vaccine should be safe. Since vaccines must be given millions of people all over the world, including young and elderly as well as immunocompromised, they must not cause itself illness or even death by the disease it is designed to prevent.

Another criterion is that vaccines must be very cost effective, if they are to be applied to large populations especially in developing countries. They should be easy to

handle e.g. avoiding a cold chain and trained personnel for administration. Furthermore, no or only few side effects should be provoked by vaccination (Ada, 1990).

1.1.2 History of vaccine development

Following the success of Jenner's and Pasteur's vaccines against smallpox (Jenner, 1798) and chicken cholera (Pasteur, 1880) or rather rabies (Pasteur, 1885), vaccine development in the early part of the 20th century took two different pathways. On the one hand, vaccinations were performed by the use of attenuated organisms with reduced pathogenicity but still able to stimulate protective immunity. However, any live vaccine can cause lethal systemic infections in the immunosuppressed and e.g. for measles and polio widespread use is prevented in developing countries due to technical and economical problems. On the other hand, vaccines based on killed inactivated organisms were tested. Killed whole-cell vaccines cannot replicate and are therefore non-infectious. But they are also less immunogenic than live vaccines and often require booster injections and additional adjuvant.

During the second half of the 20th century, detailed molecular understanding of microbial pathogenicity and protective host response towards pathogens has arisen. Together with the knowledge about the regulation of the immune system to generate effective T- and B-lymphocyte responses, a more rational approach of vaccine design was developed leading to enhanced effectiveness of vaccines that will finally fulfill the criteria of an optimal vaccine (Makela, 2000).

Classical subunit vaccines

Knowledge of the nature of individual antigens or the immune response they elicit and rapid advances in molecular biology have led to the development of subunit vaccines based on purified components of pathogens such as bacterial polysaccharides, viral surface proteins or detoxified toxins that were as effective as live whole vaccines (e.g. pertussis toxin). Pure subunits of pathogenic origin are safer to use as vaccines than live organisms, but are often very sumptuous and risky in their production due to the large-scale cultivation of pathogenic organisms. In addition, this type of non-recombinant subunit vaccines often needs coadministration of adjuvants or conjugation to protein carrier to render them more immunogenic (Liljeqvist and Stahl, 1999).

As example, many bacteria, including *Streptococcus pneumonia* and *Haemophilus* species, have an outer capsule composed of polysaccharides that are species- and type-specific for particular strains of the bacterium. The most effective defense against these pathogens is opsonization of the polysaccharide coat with antibodies. However, capsular polysaccharides elicit only a T-cell independent antibody response and are therefore not suitable in young children under the age of 2 years. To overcome this problem, bacterial polysaccharides are chemically conjugated to protein carriers, which provide peptides that can be recognized by antigen specific T cells, and converts the antibody response against the polysaccharide into a T-cell dependent response. Various conjugate vaccines have been developed against *Haemophilus influenzae* and are nowadays in use (Peltola et al., 1992; Rosenstein and Perkins, 2000).

Usually, purified antigens are not immunogenic on their own and therefore require the presence of adjuvants (e.g. alum, pertussis toxin) to improve the immunogenicity of vaccines. Adjuvants are defined as substances that enhance the immunogenicity of antigens (Ramon, 1924) by their ability to directly activate an immune response or to act as delivery system (Pizza et al., 2002). Many adjuvants provide “danger signals” and activate cells of the innate immune system, which in turn activate immune responses by secreting a wide-range of inflammatory mediators and cytokines (Medzhitov and Janeway, Jr., 1998; Aderem and Ulevitch, 2000). For example, tetanus toxoids often contain aluminum salts, which bind polyvalently to the toxoids by ionic interactions and then selectively stimulate antibody responses. In contrast, pertussis toxin has its own adjuvant properties and, when given mixed as a toxoid with tetanus and diphtheria toxoids as so called DPT triple vaccine, not only vaccinates against whooping cough but also acts as an adjuvant for the other two toxoids. In addition, adjuvants like aluminum phosphate act by depot formation at the injection site that permits a slow release of antigen. Thus, the duration of interaction between antigen and antigen-presenting cells and lymphocytes is prolonged (Glenny et al., 1931; Gupta, 1998).

Adjuvants can also modulate immune responses and facilitate the induction of cytotoxic T lymphocyte (CTL) responses against coadministered antigens. Different adjuvants may promote different types of response, for example, an inflammatory T helper 1 (Th1) response or an antibody-dominated Th2 response. Some adjuvants, like pertussis toxin, stimulate mucosal immune responses.

Moreover, coadministration of cytokines can enhance and modulate the immune response into the desired direction. For instance, coadministration of Interleukin-12 (IL-12) with a vaccine containing leishmania antigens abrogate the predominantly Th2 response in susceptible mice and generate a Th1 response leading to protection against *Leishmania major* (Scott and Trinchieri, 1997).

Recombinant subunit vaccines

Through identification of relevant protective antigens and advances in molecular biology, subunit vaccines could be established recombinantly by isolating the coding gene and transferring it to a non-pathogenic organism. The recombinant antigen is then produced by the heterologous host, thus excluding the pathogen itself, which eliminates risks associated with the production, as well as risks for contamination with toxic compounds. Furthermore, the antigen can be modified regarding optimized production of recombinant protein (high yield, easy purification, minimal proteolysis) and enhanced immunogenicity (e.g. use of fusion proteins). This resulted in the first recombinant subunit vaccine, the Hepatitis B surface antigen vaccine, produced in *Saccharomyces cerevisiae* (Valenzuela et al., 1982) that was licensed in 1986 (reviewed by Liljeqvist and Stahl, 1999).

Peptide vaccines

Chemically synthesized peptides of protective T cell epitopes against infectious diseases can elicit a strong immune response when delivered together with a carrier (Simard et al., 1997) or an adjuvant (Hsu et al., 1996). For example, immunogenical peptides can be genetically integrated into carrier proteins within a viral vector, such as hepatitis B core antigen, which are then processed *in vivo* through natural antigen-processing pathways. In contrast, ISCOMs (immune stimulatory complexes), which are lipid carriers that act as adjuvants with minimal toxicity, encourage the entry of peptides into the cytoplasm, thus enhancing the loading of peptides onto major histocompatibility complex (MHC) class I molecules (Cox et al., 1998). Synthetic peptide vaccines could be chosen to stimulate predominantly either the humoral or cellular branch of the immune response. However, a single epitope-based vaccine is not likely to be effective in an out-bred population like humans since the cellular

response will be restricted to a particular human leukocyte antigen (HLA), which will be present only in some individuals of the population.

Attenuated pathogens

Most antiviral vaccines presently in use consist of inactivated or live attenuated viruses. Thus, against polio, measles, mumps, rubella and varicella attenuated viral vaccines are used. Live attenuated viral vaccines have the advantage that they are generally more potent in eliciting relevant effector mechanisms, including cytotoxic CD8⁺ T cells, in contrast to inactivated viruses. On the other hand, they bear the risk of reverting to the virulent form. Although today, attenuation can be achieved more reliable by employing recombinant DNA techniques, minimizing the risk of reversion of the vaccine virus to a virulent strain, attenuated viral vaccines can nevertheless pose particular risks to immunodeficient recipients in whom they often behave as virulent opportunistic infections.

In contrast, most bacterial vaccines nowadays, like tetanus, diphtheria and whooping cough (caused by *Bordetella pertussis*), consist of inactivated toxins or toxoids of the corresponding bacteria. This is however limited to bacteria with toxins that are responsible for pathological conditions. Therefore, strategies to engineer genetically defined attenuations for pathogenic bacteria are also used for vaccine development (e.g. *Salmonella typhi*) (Levine et al., 2002).

Routes of vaccination

Nowadays, most vaccines are given parenteral by injection. Nevertheless, this kind of immunization suffers from several disadvantages. First, injections are expensive due to the requirement of sterile needles and syringes as well as trained personnel. They are therefore not well suited for mass vaccination in developing countries. Second, parenteral administration may not be the most effective way of stimulating an appropriate immune response at the mucosa. Most pathogens like *B. pertussis*, influenza virus, *Vibrio cholerae*, *S. typhi*, *E. coli* and *Shigella* usually enter the host through mucosal surfaces and immunity at this site is required for protection.

Therefore, efforts are undertaken to develop vaccines that can be administered via the mucosa orally or by nasal inhalation. In this respect, the use of live attenuated bacteria or viruses has been investigated for the induction of local mucosal as well as

systemic immune responses. For example, the live-attenuated polio vaccine (Sabin) consisting of three attenuated polio virus strains is highly immunogenic and effective by the oral route (Minor, 1992).

Vaccine carriers

By means of engineering genetically defined attenuated strains of bacteria and viruses which are non-pathogenic, such live vaccines are not only valuable in their own right but could also serve as vectors for the delivery of antigens from other pathogenic organisms. In the mouse, infection with *Salmonella* stimulates a powerful mucosal and systemic immune response and, in addition, can be used as carrier to stimulate the mucosal immune system against heterologous antigens. Attenuated strains of *Salmonella* have already been used successfully as carriers of heterologous antigens from organisms like *Listeria monocytogenes* (Hess et al., 1996), *Bacillus anthracis*, *L. major* (Aggarwal et al., 1990; McSorley et al., 1997), *Yersinia pestis* and *Schistosoma mansoni* (Khan et al., 1994) resulting in protection of mice against an experimental challenge with the respective pathogen.

Similarly, viral vectors like vaccinia (Mackett et al., 1982; McMahon-Pratt et al., 1993; Katz and Moss, 1997) or adenovirus (Fooks et al., 1995; Xiang et al., 1996; Mittal et al., 1996) can be engineered to carry heterologous peptides or proteins and can be used as single or combined vaccine against several different organisms. However, this type of a viral vaccine can only be used once, since long-lasting immunity is generated against the carrier that will neutralize its effectiveness upon a second administration. Prime-boost immunization strategies are therefore developed that, for instance, use DNA vaccines (see below) to prime and recombinant viral vectors as a booster. Improve of immunity against HIV in a primate model (Letvin et al., 1997; Robinson et al., 1999; Fuller et al., 1997), malaria (Sedegah et al., 1998) and HSV (Eo et al., 2001) was elicited this way.

Novel experimental approaches

In novel approaches, plant viruses, which are non-pathogenic in humans, were employed as vaccine vectors. Such viruses can be engineered to incorporate heterologous antigens into transgenic plants for direct mucosal delivery as food (Haq et al., 1995; Tacket et al., 1998; Arakawa et al., 1998).

An unexpected highly versatile new vaccination strategy was discovered recently. (Wolff et al., 1990; Tang et al., 1992). Isolated plasmid DNA encoding a foreign protein under the control of a suitable promoter was injected directly into muscle or skin by syringe or applied as DNA coated gold beads by gene gun. Uptake of the DNA by cells of the host causes expression of the plasmid encoded protein (Williams et al., 1991; Wolff et al., 1990). This has been shown to engender both cellular and humoral immune responses in a variety of rodent and primate disease models (reviewed by Donnelly et al., 1997). Recently, several DNA vaccines have entered human clinical trials for various diseases, including influenza, hepatitis B, HIV, malaria as well as cancer (reviewed by Srivastava and Liu, 2003). The predominant ability of DNA vaccines to generate cellular immune responses may be crucial for the design of completely novel and/or more effective vaccines against intracellular organisms that only can be fought by cell-mediated immunity such as *Mycobacterium tuberculosis*, *Plasmodium falciparum*, *Leishmania* and HIV. In contrast, currently available vaccines against most viruses and bacteria are based on the induction of a long-lived humoral antibody production against the pathogen.

As extension, the use of bacteria as delivery system for antigen-encoding plasmid DNA has recently been shown to provide a highly efficient alternative to intramuscular or intradermal immunization of DNA. Using bacteria for oral delivery of DNA vaccines combines the advantages of the adjuvant properties of bacterial vectors, the selectivity to target antigen-presenting cells and immune-inductive sites, with the versatility of DNA vaccines. Thus, it stimulates efficiently humoral, T helper and cytotoxic responses, systemically as well as mucosally. Extremely promising results have been already obtained using attenuated *Salmonella* strains (Darji et al., 1997, 2000; Weiss, 2003) or invasive intracellular pathogens such as *Shigella spp.* (Sizemore et al., 1995).

1.2 DNA vaccination

The use of DNA plasmids to induce immune responses by direct injection of DNA that encode antigenic proteins has been termed DNA vaccination or genetic immunization (Wolff et al., 1990; Tang et al., 1992). DNA vaccines employ genes encoding proteins of pathogens, rather than the protein or an attenuated version of the pathogen itself. Since the 1990s it has been shown that this method elicits cell-

mediated immune responses and protective antibodies in a number of animal models for viral, bacterial and parasitic diseases (Donnelly et al., 1997), but has also been effective in tumor models and to ameliorate allergic reactions (reviewed by Gurunathan et al., 2000).

DNA vaccines generally consist of a bacterial plasmid with an origin of replication (e.g. pUCori) suitable for high yields of plasmid and an antibiotic resistance gene to confer antibiotic-selected growth in *E. coli*. In addition, the plasmid includes a strong viral promoter (e.g. human cytomegalovirus immediate/early promoter (Boshart et al., 1985)), the gene of interest and an mRNA transcript termination/polyadenylation sequence (e.g. from SV40 (Pfarr et al., 1986)) for directing expression in mammalian cells (Williams et al., 1991; Wolff et al., 1990). The plasmid is grown in bacteria (*E. coli*), purified, dissolved in saline solution and then injected into the host.

Upon injection, protective antigen from the particular pathogen is expressed. After processing of this endogenous antigen, presentation of antigenic peptides bound to MHC class I molecules then initiate activation of cytolytic T cells (Ulmer et al., 1993). In addition, T helper cells and B cells are stimulated, which consequently results in protection to a challenge with pathogenic microorganisms and tumors (Xiang et al., 1994; Sedegah et al., 1994). How these immune responses are generated, still remains unclear. Due to the relative small amounts of proteins synthesized during DNA vaccination, the most likely explanation for the efficient induction of a broad and sustained immune response is the immune-enhancing properties of the DNA itself and/or the type of antigen presenting cells (APC) transfected. There are at least three mechanisms by which antigens encoded by plasmid DNA could be processed and presented to the immune system: a) direct priming by somatic cells (e.g. myocytes, keratinocytes); b) direct transfection of professional APCs (e.g. dendritic cells (DCs)); and c) cross-presentation.

In several studies, it was demonstrated that bone marrow-derived APCs, rather than myocytes or keratinocytes, mediate cellular immune responses (including CD4⁺ and CD8⁺ T cells) after DNA vaccination (Corr et al., 1996; Doe et al., 1996; Iwasaki et al., 1997; Torres et al., 1997). Moreover, injection into muscle (Porgador et al., 1998; Akbari et al., 1999) as well as gene gun vaccination into skin (Condon et al., 1996) leads to direct transfection of DCs that can present antigen efficiently to T cells. Somatic cells apparently express only low amounts of MHC class I molecules, but neither MHC class II (Hohlfeld et al., 1991) nor costimulatory molecules such as B7.1

or B7.2. Therefore, they need to transfer the antigen encoded by the DNA vaccine to professional APCs by a process called cross-presentation, in order to induce T cells (Fu et al., 1997). Until now, this mechanism has been only partly elucidated. During cross-presentation secreted proteins or peptides from somatic cells and/or professional APCs can be taken up by neighboring DCs via phagocytosis of either apoptotic or necrotic bodies. In turn, the exogenous antigens were directly transferred to the cytosol and can be presented to T cells in the context of MHC class I as well as MHC class II. Processing of antigens via MHC class I and MHC class II pathways could directly stimulate naïve CD8⁺ T cells and CD4⁺ T cells, respectively (Heath and Carbone, 2001). Although the immune response elicited by genetic immunization is weak compared to that induced by traditional vaccines, it is exceptionally long lasting (Davis et al., 1994; Xiang et al., 1994).

Genetic vaccination offers several advantages over traditional vaccines and potential solutions for current problems in vaccination:

- A major attribute of DNA vaccines is their ability to deliver genes into cells for generation of MHC class I restricted cytolytic T lymphocyte responses. Ulmer could demonstrate that DNA vaccination against influenza provides broader protection against different strains of the virus (Ulmer et al., 1993). This was due to the generation of cytotoxic T lymphocytes that recognized epitopes from conserved proteins inside the virus capsid or proteins that are only expressed during the infection cycle. In contrast, antibodies are normally directed against surface or envelope proteins.
- The outstanding feature of DNA vaccines to induce MHC class I-restricted cytotoxic T lymphocytes (CTLs) against entire proteins (rather than peptides) allows determinant selection to occur in the host. Thereby, DNA vaccines are efficacious across the diversity of the MHC haplotypes, as has been already shown in mice immunized with nucleoprotein from influenza A virus (Ulmer et al., 1993), hepatitis B surface and core protein (Schirmbeck et al., 1995; Kuhrober et al., 1997), and HIV env and gag antigens (Shiver et al., 1995, 1997; Liu et al., 1996; Boyer et al., 1998; Thomson et al., 1998).
- Induction of antigen specific cytolytic T cell responses by DNA vaccination has likewise been observed in non-human primates (Donnelly et al., 1995; Liu et al., 1996; Amara et al., 2001; Barouch et al., 2001) and in human clinical trials (Wang et al., 1998). Thus, it demonstrates its efficacy in outbred populations.

Moreover, cellular responses elicited upon genetic immunization were demonstrated to be protective in models of influenza virus (Ulmer et al., 1993), lymphocytic choriomeningitis virus (Yokoyama et al., 1995; Martins et al., 1995), and herpes simplex virus (Manickan et al., 1995; reviewed by Srivastava and Liu, 2003). Accordingly, DNA vaccines offer a simple alternative to conventional methods to generate CTLs, such as immunization with peptides or live attenuated viruses.

- Expressing the endogenous synthesized antigen with native posttranslational modifications (e.g. glycosylation pattern), conformation, and oligomerization to elicit antibodies of optimal specificity (particular advantageous for viral proteins) have contributed to protection in preclinical disease models against challenge with the relevant infectious pathogen like influenza (Ulmer et al., 1993, 1994), rabies virus (Xiang et al., 1994) hepatitis B virus (Prince, 1996), *M. tuberculosis* (Huygen et al., 1996; Tascon et al., 1996) or *L. major* (Xu and Liew, 1995). In addition, DNA vaccines stimulate helper T cell responses which contribute to the long-lasting immune response (Donnelly et al., 1997).
- Vectors for genetic immunization are easily to construct and the genes inserted into a plasmid can be modified readily, allowing removal or insertion of transmembrane domains, signal sequences or residues effective in protein processing.
- Certain sequence motifs, such as CpG motifs, in bacterial plasmid DNA can function itself as adjuvant or immunomodulator (Krieg et al., 1995a; Sato et al., 1996).
- Altering the nucleotide sequence of the vector may affect the immunogenicity of DNA vaccines (Yamamoto et al., 1992; Krieg et al., 1995a; Sato et al., 1996).
- The stability of genetic vaccines as well as their heat resistance eliminates the need for a cold chain, which is very important for the use in developing countries.
- The DNA can be detected during a long period of time, indicating that it acts as antigen depot (Zhu et al., 1993). Thereby, genetic vaccines induce long-term memory for T and B cells and can provide upon a single inoculation long-lasting protective immunity (Ertl and Xiang, 1996).

- DNA vaccines do not carry any risk of converting to virulence (Ertl and Xiang, 1996).
- The plasmids are constituted without an origin of replication that is functional in eukaryotic cells. Such plasmids neither replicate in the mammalian host nor should they integrate into the chromosomal DNA of the animal (Donnelly et al., 1997).
- The co-expression of cytokines or costimulatory molecules can enhance the immune response and offers the possibility of modulating the induction of an immune response into the desired direction (Xiang and Ertl, 1995; Geissler et al., 1997; Kim et al., 1997).
- DNA vaccines, given intramuscularly and formulated in saline, elicit a Th1-like helper T cell response, whereas intradermal vaccination using a gene gun appears to bias the immune response towards Th2-like responses (Williams et al., 1991; Tang et al., 1992; Feltquate et al., 1997). In addition, the quantity of the plasmid DNA administered and the method and site of its introduction may significantly influence the types of immune responses generated against the proteins encoded by the plasmid.
- The combination of diverse immunogens into a single preparation facilitates simultaneous immunization for several diseases.

As already mentioned, the induced immune response is comparably weak. Thus, the DNA has to be inoculated several times and possibly with additional adjuvants. On the other hand, DNA vaccines are very effective in preclinical animal models, demonstrating the robustness of the technology. Nevertheless, there may be a need for improved formulations of DNA vaccines in human clinical trials that can lower the dose of DNA required for efficacy, that are more stable, or that transfect a greater number of cells or deliver a greater amount of DNA into cells.

To account for these problems, prime-boost protocols have been developed (as mentioned above). In addition, targeting of certain tissues or cells for transfection by DNA vaccines may be desirable, particularly for cellular and mucosal responses (Ulmer et al., 1996). In this respect, the discovery to orally deliver plasmid DNA with attenuated enteric bacteria such as *Salmonella*, *Shigella* and *Listeria* has raised great interest and provoked extensive research activities.

1.3 Bacteria as live antigen delivery systems

Since most pathogens usually enter the host through mucosal surfaces, the traditional way of injecting vaccines with needles may be not the most effective way to stimulate an appropriate immune response. Therefore, new vaccines that could be administered to the mucosa orally or by nasal inhalation are now designed. In addition, they should be delivered efficiently and selectively to antigen-presenting cells as well as modified for targeting them particularly into antigen-processing pathways within the cell.

An understanding of mechanisms of mucosal immunity has led to the development of techniques to target antigens to microfold (M) cells that overlay Peyer's patches.

- M cell targeting with nonliving vaccines has proved difficult because unprotected macromolecules are readily digested in the intestine. Consequently, one approach has been to package antigens in microparticels that provide protection from intestinal enzymes and to take advantage of the fact that M cells can endocytose particles up to several μm in size (Eldridge et al., 1991). However, many of such microparticles, including liposomes, which also have been tested in this context, adhere to mucosal surfaces by hydrophobic interactions, but uptake by M cells is inefficient.
- Macromolecules or particles can be conjugated or coated with ligands, such as CTB (cholera toxin subunit B), that allow passage through mucous gels and adherence to M cells. Nevertheless, accessibility to membrane receptors is a limiting factor (Frey et al., 1996).
- ISCOMs consisting of cholesterol, phospholipids, cell membrane antigens and saponins (Cox et al., 1998) together with the immunogen has been demonstrated to induce cellular and humoral responses as well as local antibody responses after either intranasal or oral administration (Morein and Bengtsson, 1998; Mowat et al., 1999; Smith et al., 1998).
- Oral application of DNA encapsulated into cochleates was shown to induce strong CTL and antibody responses (Mannino et al., 1998). Cochleates are rigid calcium-induced spiral bilayers of anionic phospholipids with a unique structure that are relatively stable after lyophilization or in harsh environments.

Despite of such promising approaches, mucosal delivery via inert carriers misses the efficiency required for a generally applicable vaccine. Exploiting pathogens that can target themselves to M cells and enter the mucosa at immune inductive sites can bypass these difficulties. Thus, invasive intracellular bacteria have been recognized, besides being carriers of heterologous antigens, to be efficient vehicles for the mucosal delivery of genes encoding protective antigens. Several advantages are associated with their use:

- Attenuated live bacterial vaccines could prove considerably less expensive than killed preparations. Further, they are relatively economical in their manufacture and easy to conserve compared to tissue culture based, conjugated and purified subunit vaccines.
- The employment of live attenuated bacteria as mucosal (oral or intranasal) rather than parenteral vaccine simplifies the administration (e.g. the use of sterile syringes is not required), which could avoid needle-stick transmission of pathogens (e.g. HIV), mainly in developing countries.
- They could be expected to cause fewer side effects than traditional whole-cell enteric vaccines (e.g. killed typhoid vaccine) and the possibility to administer live vaccines as enteric capsules containing lyophilized organisms should ideally eliminate the need for a cold chain.
- In contrast to oral subunit vaccines, that need coadministration of adjuvants like the cholera toxin of *V. cholerae*, the heat-labile enterotoxin from *E. coli* (LTB) or aluminum salts to induce efficacious immune response, live replicating bacterial vectors produce their own immunomodulating factors (e.g. cell wall components) *in situ*.

As in the case of a natural infection with wild type organisms, mucosal immunization e.g. with attenuated *Salmonella* and *Shigella* stimulates every arm of the immune system. Consequently, attenuated *Salmonella* and *Shigella* constitute highly versatile live carriers for delivering heterologous antigens to the immune system. Such antigen could be expressed by the bacteria themselves by means of an open reading frame encoded by a prokaryotic expression plasmid or chromosomally integrated. Alternatively, eukaryotic expression plasmids (i.e. DNA vaccines) can be employed that are delivered to antigen-presenting cells for gene expression and immune stimulation.

1.3.1 Attenuated live bacteria as combined vaccines

Both Gram-negative and Gram-positive bacteria, including mycobacterial strains have been investigated for delivery of foreign antigens. The particular antigen-encoding DNA fragment can be inserted into the attenuated carrier by recombinant DNA techniques, either for chromosomal expression or introduced as prokaryotic expression system on a plasmid. Such live bacterial carriers have been extensively studied as mucosal vaccines, both against the corresponding disease caused by the wild type strain of the particular carrier as well as delivery system against diseases provoked by other pathogens. This vaccination strategy is based on the fact that the attenuated bacteria establish a limited infection, resembling early stages of infections generated by its wild type counterpart. This leads to the induction of natural immune reactions in the host to the carrier itself as well as their cargo.

Live recombinant Gram-negative bacteria such as *Salmonella enterica* spp. (Sadoff et al., 1988; Clements et al., 1986; Tacket et al., 1990, 2000), *Shigella flexneri* (Ryd et al., 1992; Klee et al., 1997), *Vibrio cholerae* (Schodel et al., 1991; Acheson et al., 1996; Lang and Korhonen, 1997), *Yersinia enterocolitica* (Sory and Cornelis, 1990), and *Bordetella pertussis* (Mielcarek et al., 1998) have been studied in animal models and human clinical trials as potential vaccine delivery systems for heterologous antigens. So far, *Salmonella* spp. and *S. flexneri* have been employed systematically as carriers.

Genes encoding enzymes for production of polysaccharide antigens as well as protein antigens from various genera of bacteria, viruses and even eukaryotic parasites have been expressed in attenuated strains of *Salmonella* spp. either on plasmids or by genes integrated into the chromosome. Following mucosal immunization with these live vectors, specific serum IgG antibodies, secretory IgA (sIgA) mucosal antibody or cell-mediated immune responses were stimulated and conferred protection against a challenge with wild type organisms or toxins (reviewed by Levine et al., 2002).

Although this *Salmonella* system is versatile, it has limitations. For example, *Salmonella* cannot glycosylate proteins. If glycosylation as post-translational modification of protective epitopes within viral proteins is required for correct folding of the antigen to stimulate specific antibodies against conformational epitopes, *Salmonella* can not be the vector system of choice (Levine et al., 2002).

1.3.2 Attenuated live bacteria for the delivery of DNA vaccines

In the last few years, it has been demonstrated that attenuated intracellular bacteria can be used also as vehicle to efficiently target DNA vaccines to professional antigen presenting cells such as macrophages and DCs *in vitro* as well as *in vivo*.

The concept of transkingdom transfer of eukaryotic expression plasmids between bacteria and mammalian cells has been first described by Schaffner (1980) several years ago, which was improved by the fusion between eukaryotic cells and protoplasts of plasmid carrying *E. coli*. By now, DNA-transfer between, Gram-negative and Gram-positive bacteria (Trieu-Cuot et al., 1993; Charpentier et al., 1999), between bacteria and yeast (Heinemann and Sprague, Jr., 1989), as well as bacteria and plants (Lessl and Lanka, 1994) is well established. More recently, transfer of DNA vaccines from viable bacteria to mammalian host cells has been discovered by four groups independently using attenuated strains of *Shigella flexneri*, invasive *E. coli* or *Salmonella typhimurium* (Sizemore et al., 1995; Darji et al., 1997; Courvalin et al., 1995; Powell et al., 1996). In the meantime, the number of bacteria that are able to transfer expression plasmids to mammalian cells *in vitro* and *in vivo* has been multiplied. The Gram-negative species *S. typhi*, *S. choleraesuis*, *Yersinia pseudotuberculosis*, *Y. enterocolitica* and the Gram-positive strain *L. monocytogenes* were successfully applied (Weiskirch and Paterson, 1997; Shiau et al., 2001; Dietrich et al., 1998, 2001; Hense et al., 2001; Al Mariri et al., 2002).

Most intracellular bacteria can be transformed easily with DNA vaccine vectors. Accordingly, attenuated mutants still equipped with adhesion and invasion properties, can carry these plasmids selectively into the phagosome (*Salmonella*, invasive *E. coli*) or cytosol (*Shigella*, *Listeria*) of APCs, where they undergo lysis due to their metabolic attenuation, an inducible autolytic mechanism or treatment with appropriate antibiotics. Upon liberation of the eukaryotic expression plasmid due to lysis and translocation into the nucleus of the host cell, the encoded antigen is expressed and can subsequently induce humoral and cellular immune responses against bacterial, viral and tumor antigens conferring protection against bacterial and viral infection and tumor challenge (Weiss and Krusch, 2001; Weiss and Chakroborty, 2001). Mucosal application either nasally or orally of such recombinant bacteria additionally leads to transfection of the gut-associated lymphoid tissue, which induces efficient mucosal responses. This type of immunization was more

efficacious than either direct application of antigen, vaccination with naked DNA or using the same bacterium as a heterologous carrier expressing the antigen via a prokaryotic promoter (Weiss and Krusch, 2001; Weiss and Chakraborty, 2001).

Delivering plasmids for genetic immunization with an attenuated bacterial carrier can combine the advantages and versatilities of both systems as already mentioned above. Moreover, the amount of plasmid required for bacterial-mediated DNA vaccination is astonishingly small compared to needle injection of naked DNA. This is most likely based on bacterial properties to target inductive sites of the immune system whereupon the antigen is expressed in immunologically relevant cells. Furthermore, bacterial cell wall components (e.g. lipopolysaccharides (LPS) from Gram-negative or lipoteichoic acids from Gram-positive bacteria) and unmethylated cytosine-phosphate-guanosine (CpG) motifs in the expression plasmid and/or bacterial chromosomal DNA provide their own adjuvant activity and can enhance and/or modulate immune responses (Krieg, 1999; Aderem and Ulevitch, 2000). In addition, these live bacterial vaccine strains can also deliver plasmids encoding therapeutic molecules (e.g. cytokines) or genes to complement genetic defects.

In comparison to currently used viral and non-viral vectors, bacterial gene delivery vehicles include additional advantages. First of all, bacteria are cost-effective to produce, easy to store and to transport. The tissue tropism of bacteria can be employed to target genes to certain organs and the ability of some bacteria to spread from cell-to-cell might render them capable of targeting tissue layers inaccessible to other vector systems. Moreover, they are controllable by common antibiotics.

However, bacteria-mediated gene transfer is yet confined mainly to preclinical studies either *in vitro* or in animal models. So far, gene transfer *in vivo* attempts were primarily directed towards vaccination strategies using *Shigella flexneri* or *Salmonella spp.* as carrier in experimental models (Darji et al., 1997, 2000; Flo et al., 2001; Fennelly et al., 1999; Pasetti et al., 1999; Shata et al., 2001), but also in clinical trials (Cunningham and Nemunaitis, 2001). *Salmonella*-mediated mucosal DNA vaccination has been extensively investigated using attenuated *S. typhimurium* as transfer system, but also *S. typhi* and *S. choleraesuis* have been tested in this context.

1.3.3 Main bacterial species employed for DNA transfer

S. typhimurium

This strain will be described in more detail below.

S. typhi

The strain *S. typhi* Ty21a (Germanier and Fuer, 1975), which is attenuated by a defect in the galactosidase degradation pathway due to a mutation in *galE*, is already licensed as typhoid fever vaccine for humans. Therefore, it was thought that attenuated strains of *S. typhi* cannot only serve as effective oral vaccines to prevent typhoid fever in humans (Wahdan et al., 1982; Levine et al., 1987, 1999), but also as live vectors to deliver foreign antigens to the immune system, either by the bacterial expression of antigens through prokaryotic expression plasmids or by delivery of foreign genes carried on DNA vaccines (Levine et al., 1997; Dietrich et al., 2000; Garmory et al., 2002). Since *S. typhi* is not virulent in mice, a proof of principle using these bacteria as carrier for DNA vaccination is limited to nasal or intraperitoneal administration. In the meantime, several genetically defined mutant strains were additionally developed like the strain CVD 908-*htrA* (*aroC*, *aroD* and *htrA* mutant) (Tacket et al., 2000) or CVD 915, which is attenuated by mutation in *guaBA* leading to a defect in the guanine biosynthesis pathway (Wang et al., 2001).

Intraperitoneal application of *S. typhi* Ty21a carrying a plasmid encoding the nucleoprotein of measles virus to mice resulted in specific CTL responses (Fennelly et al., 1999). In a comprehensive study using a fragment of tetanus toxin, nasally administered *S. typhi* CVD 915 carrying a eukaryotic expression plasmid elicited higher antibody levels than did *Salmonella* carrying a prokaryotic expression plasmid or just intramuscular injection of the naked DNA vaccine (Pasetti et al., 1999). Interestingly, ampicillin treated mice showed low antibody responses after oral immunization with the Ty21a strain carrying a plasmid encoding the identical fragment of tetanus toxin (Woo et al., 2000). This suggests that *S. typhi* might enter via the intestine, when the animals are devoid of competing commensal bacteria.

Shigella flexneri

The Gram-negative bacterium *S. flexneri*, which cause shigellosis in humans, enters the cytosol of host cells after phagocytosis and can deliver the plasmids directly to this intracellular compartment. Different metabolically attenuated mutants of *S. flexneri* have been demonstrated to successfully deliver plasmid DNA *in vitro* (Sizemore et al., 1995; Courvalin et al., 1995; Noriega et al., 1994, 1996; Powell et al., 1996). Since mice are not susceptible to gastrointestinal infection with *S. flexneri*, intranasal application was used to investigate its potential as carrier for DNA vaccines in these animals. Such *Shigella*-mediated gene-transfer *in vivo* has induced strong cellular and humoral immune responses against several antigens (Sizemore et al., 1995, 1997; Fennelly et al., 1999).

E. coli

Non-pathogenic strains of *E. coli* can also be employed as vehicles for DNA vaccination provided that they are rendered invasive (Courvalin et al., 1995; Grillot-Courvalin et al., 1998, 1999). This system is mainly used *in vitro* so far, but also *in vivo* data were obtained more recently (Shiau et al., 2001; Radford et al., 2002, 2003).

Listeria monocytogenes

L. monocytogenes is a Gram-positive, intracellular pathogen that enters the cytosol of the host cell. Attenuated strains of *L. monocytogenes*, defective in intracellular mobility and cell-to-cell spread (Dietrich et al., 1998), have been shown to be very effective delivery systems for targeting the heterologous antigens to both MHC class I and class II presentation pathways (Weiskirch and Paterson, 1997; Jensen et al., 1997; Guzman et al., 1995). The ability to stimulate the production of Th1-type cytokines make these bacteria especially attractive as delivery system for the development of vaccines against viruses, tumors and intracellular parasites (Pan et al., 1995; Slifka et al., 1996; Jensen et al., 1997; Ikonomidis et al., 1997; Paglia et al., 1997). So far, efficient DNA transfer was only demonstrated *in vitro* in phagocytic and non-phagocytic cells (Hense et al., 2001; Krusch et al., 2002; Pilgrim et al., 2003).

1.4 Immunization with *Salmonella typhimurium aroA*⁻ as carrier

Effective protection against invasive *Salmonella* infections in man and animals is best elicited by live attenuated organisms rather than by killed vaccines (Collins, 1974). In addition, the proven ability to induce potent cell-mediated immunity, as well as humoral and local secretory responses to recombinant foreign antigens, makes these attenuated *Salmonella* particularly attractive as vehicle for the oral delivery of antigens from a variety of infectious microorganisms (Hormaeche, 1991). Moreover, attenuated *S. typhimurium* strains have been extensively studied as live vaccine carriers in mice as a model for the prediction of responses that would occur when humans are immunized orally with homologous strains of *S. typhi*.

Salmonella typhimurium

S. typhimurium is a Gram-negative, facultative anaerobic rod that belongs to the group of enterobacteria. Through liberation of endotoxin (lipopolysaccharides of the bacterial cell wall), which cause irritation of the mucous membrane, these facultative intracellular pathogen causes gastroenteritis (Schlegel, 1992). In the mouse model, *S. typhimurium* is the causative agent of murine typhoid fever and serves as experimental model for human typhoid caused by *S. typhi* (Collins, 1974; Hormaeche, 1979).

Because of the extensive knowledge on the molecular genetics and physiology of many strains, *Salmonella* spp. are particularly suited as delivery system for DNA vaccines. In addition, a large body of documentation already exists pertaining to their utility as heterologous antigen carriers capable of inducing protective immune responses (Newton et al., 1989; Fairweather et al., 1990; Roberts and Socket, 1994; reviewed by Chatfield et al., 1994). Safe attenuated strains of *Salmonella* are available and are already in use as vaccines in man and farm animals (Germanier and Fuer, 1975; Hassan and Curtiss, III, 1996; Steinbach et al., 1996; Fox et al., 1997; Mastroeni et al., 2000).

A fundamental feature of pathogenesis of *Salmonella* that contributes to their success as live vector is their ability to invade the intestinal mucosa. To induce a mucosal immune response or a systemic infection, orally applied *Salmonella* first have to pass across the mucosal surfaces of the gastrointestinal tract. Lymphoid follicles in mucosal lymphoid tissues of the small intestine and colon are covered by

the follicle-associated epithelium (FAE). This FAE contains M cells, a unique epithelial cell type specialized for transepithelial transport of macromolecules, particles, and microorganisms (Neutra et al., 1996a,b; Clark et al., 1994) (Fig. 1.1). Such specialized cells lack the mucin barrier, the rigid brush border cytoskeleton and digestive properties of other mucosal epithelial cells. Instead, they can bind and endocytose macromolecules and microorganisms, which are then transcytosed intact and delivered to the underlying lymphoid tissue. The M cell basolateral membrane is deeply invaginated to form a large intraepithelial pocket containing T lymphocytes

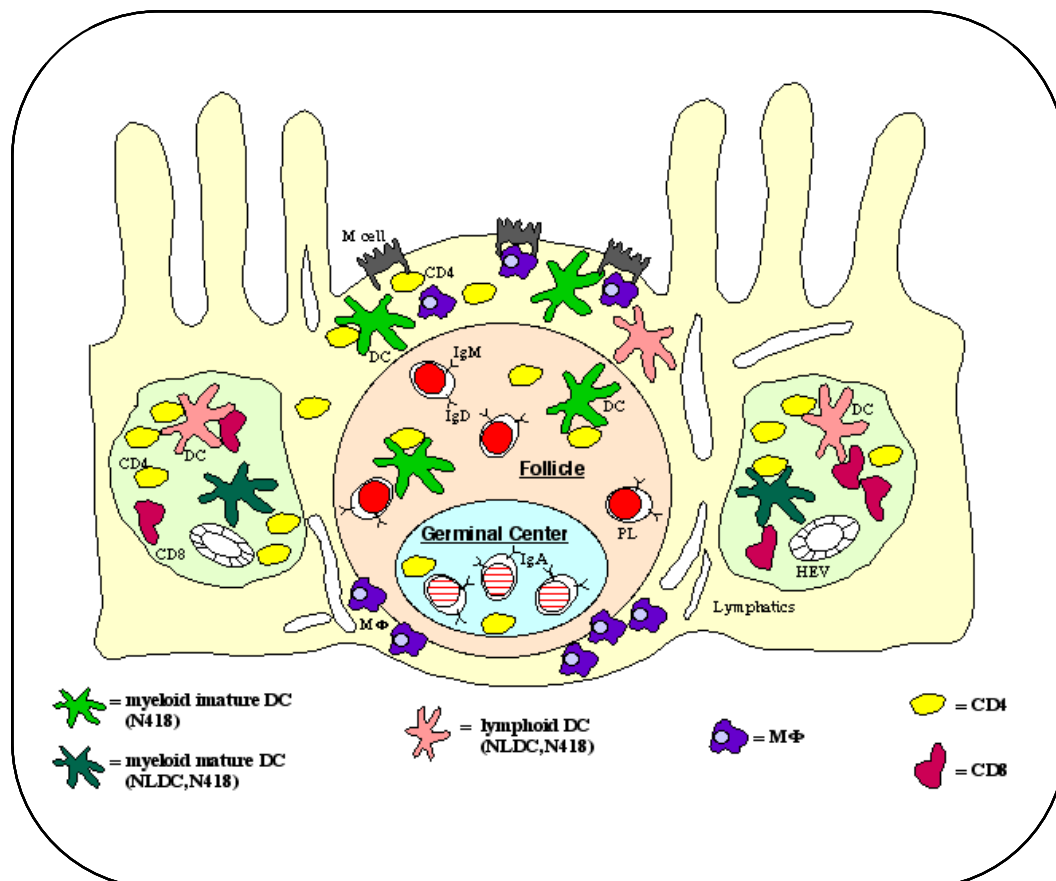


Fig. 1.1: Structural features of Peyer's Patches. The lymphoid follicle of Peyer's patches is covered by the follicle-associated epithelium (FAE) that contains M cells specialized for transepithelial transport of macromolecules, particles and microorganisms. The underlying follicle consists of large central dome of B lymphocytes surrounded by smaller numbers of T cells.

(including CD4⁺ helper cells and CD45RO⁺ memory cells), B lymphocytes, macrophages (Neutra et al., 1996a) and DCs (Hopkins et al., 2000). Several pathogenic bacteria causing systemic infections, including *Salmonella*, target these M cells to gain access to the body (Neutra and Kraehenbuhl, 1992). The main entrance of invasive *S. typhimurium* are the Peyer's patches (PP) of the ileum, appearing as aggregated mucosal lymphoid follicles, and possibly the lymphoid patches of the appendix (Carter and Collins, 1974; Hohmann et al., 1978). In addition, transepithelial invasion of enterocytes has also been observed (Ohl and Miller, 2001).

Jones et al. (1994) showed that *Salmonella* adhere rapidly and selectively (but not exclusively) to M cells. The following invasion initiates signal transduction events that are associated with loss of microvilli (Takeuchi, 1967; Kohbata et al., 1986) and cause active ruffling of the M cell apical surface. Further, these modifications are accompanied by rapid disassembly of the apical cytoskeleton *in vivo* as well as *in vitro* (Bliska et al., 1993; Finlay et al., 1991; Francis et al., 1992, 1993; Jones et al., 1993, 1994; Clark et al., 1994; Neutra et al., 1996a). Such events finally result in engulfment of bacteria by macropinocytosis into membrane-bound vesicles (Takeuchi, 1967; Neutra et al., 1996a,b; Clark et al., 1994). Rapid transcytosis follows and *Salmonella* are transported into the intraepithelial pocket. Thus, they directly reach the organized mucosa-associated lymphoid tissue (MALT), the inductive site of mucosal immune responses (Neutra and Kraehenbuhl, 1994). There, the *Salmonella* are taken up by professional phagocytes (including macrophages and DCs), which induce local secretory responses through activation of IgA secreting B cells and probably elicit systemic responses after migration into lymphnode and spleen.

S. typhimurium strains that are commonly used as live vectors have been attenuated by mutations in:

- *cya* (encoding adenylate cyclase) and *crp* (encoding the cyclic AMP receptor protein), that together comprise a global regulatory system affecting multiple virulence and housekeeping genes (Curtiss, III and Kelly, 1987);
- *aroA*, *aroC* or *aroD*, encoding enzymes in the biosynthesis of aromatic metabolites (Dogan et al., 1988);
- *phoP*, *phoQ*, a regulatory system controlling genes that allow *Salmonella* to survive within phagolysosomes in macrophages (Miller et al., 1989);

- htrA, encoding a stress response protein that functions as a serine protease (Chatfield et al., 1992b).

Such attenuated *Salmonella* complete the early steps in pathogenesis. They gain access to APCs in the gut-associated lymphoid tissue (GALT) via M cells of the Peyer's patches (Neutra et al., 1996a; Siebers and Finlay, 1996) and migrate into lymphnodes and spleen. But they do not cause adverse clinical responses or detectable bacteremia. With this kind of delivery, cell-mediated, humoral and local secretory immune responses of both mucosal as well as systemic immunity are induced.

***S. typhimurium aroA*⁻**

Salmonella, unlike vertebrates, cannot assimilate exogenous folate and have to synthesize it from *p*-aminobenzoic acid (*p*AB). 2, 3-dihydroxybenzoate (DHB) is the precursor of enterocholin, the iron-aquisition compound of gram-negative bacteria. *Salmonella* synthesize *p*AB and DHB from chorismate, the final product of the aromatic biosynthetic (*aro*) pathway (Hoiseth and Stocker, 1981). Lesions in the gene *aroA* cause a complete block at any step of the chorismate pathway rendering *S. typhimurium* auxotrophic for these two compounds, which are not available in vertebrate tissues. Such auxotrophic mutants are non-virulent in the immuno-competent host (Bacon et al., 1950, 1951; Yancey et al., 1979).

The *aroA* gene encodes the enzyme 5-enolpyruvylshikimate-3-phosphate synthase, vital for a functioning chorismate pathway and essential to bacteria for the biosynthesis of the aromatic ring (Pittard et al., 1990). Insertion of the transposon Tn10 into the gene *aroA* (Kleckner et al., 1975; Kleckner, 1977) generates aromate-requiring derivatives of virulent *S. typhimurium* strains. The transposon Tn10 encodes the tetracycline-resistance gene and has the tendency to cause DNA alterations within the transposon itself. Many such deletion or deletion-inversion events cause both, inability of the affected gene to revert to the wild-type form and loss of tetracycline resistance (Hoiseth and Stocker, 1981).

These live attenuated *Salmonella* have a very limited invasive potential, grow slowly *in vivo* and finally die due to their attenuation. In this way, they just cause a mild, low-grade infection, after which bacteria are cleared by the immune system from the tissues (Hormaeche, 1991). However, they can survive long enough to induce an efficacious immune response (Hoiseth and Stocker, 1981; Stocker, 1988). Mice

which received such attenuated *S. typhimurium* as live vaccine are protected against challenge with a virulent strain (Dougan et al., 1994). Moreover, live *Salmonella typhimurium aroA⁻* directs the cell-mediated immune response into a Th1 response, which is advantageous for clearance of intracellular bacteria (Darji et al., 1997; VanCott et al., 1998). Administration of *aroA* mutant strains is more efficacious than vaccination by injection of killed *Salmonella*, which confers only poor protection against reinfection with a virulent *Salmonella* strain (Robson and Vas, 1972). Similar results were observed in cattle (Smith et al., 1984; Jones et al., 1991), sheep (Mukkur et al., 1987) and chickens (Barrow et al., 1990; Cooper et al., 1990).

Microorganisms with genetically defined, non-reverting mutations in attenuating genes are considered to be safe live vaccines since they have been engineered to require essential metabolites not available in vertebrate tissues. For example, attenuation due to a requirement for an unavailable essential metabolite could be a safety factor if *aro* mutants were administered to subjects with defective immunity (Hormaeche, 1991). However, *S. typhimurium aroA⁻* was shown to be partly virulent, since it is still lethal in mice in which interferon γ has been deleted (Paglia et al., 2000). Safety of live vaccines can further be increased by introducing more than one attenuating lesion (Dougan et al., 1988; Miller et al., 1989; Fairweather et al., 1990). Since *Salmonella* strains are highly immunogenic, if they are administered orally, live attenuated *Salmonella* vaccines can serve not only as oral vaccines against invasive salmonellosis in man and animals but are also attractive for the development of multivalent vaccines, which are based on the expression of heterologous antigens in these strains (Chatfield et al., 1995).

1.5 *S. typhimurium aroA⁻* as carrier of heterologous antigens

The *aroA⁻* mutants of *S. typhimurium* with genetically defined attenuations are safe live vaccines and can serve as vehicles for heterologous antigens of viruses, parasites, bacteria and other immunogens. In mouse infection models, recombinant *S. typhimurium aroA⁻* were successfully employed for hepatitis B (Schodel et al., 1994a; Hopkins et al., 1995; Londono et al., 1996), herpes simplex (Chabalgoity et al., 1996), malaria (Sadoff et al., 1988; Schodel et al., 1994b; Haddad et al., 1995), *Shistosoma mansoni* (Khan et al., 1994), tetanus (Fairweather et al., 1990; Chatfield et al., 1992a; Dunstan et al., 1998) and streptococcus (Redman et al., 1996;

Hajishengalis et al., 1996). Expression of HIV antigens by *S. typhimurium* was shown to induce specific immune responses in mice (Charbit et al., 1993; Fouts et al., 1995).

1.6 Oral genetic vaccination with *Salmonella aroA*⁻

The use of *S. typhimurium* as carrier for oral transgene vaccination was first demonstrated with the two virulence factors listeriolysin O (LLO) and ActA of *L. monocytogenes*, and β -galactosidase of *E. coli* as antigens in the mouse model (Darji et al., 1997). In the meantime, oral DNA vaccination with *S. typhimurium* as carrier has been successfully extended to *Chlamydia trachomatis* (Brunham and Zhang, 1999), herpes simplex virus 2 (Flo et al., 2001), hepatitis B virus (Woo et al., 2001; Zheng et al., 2001), and hepatitis C virus (Wedemeyer et al., 2001), *Yersinia pestis* (Garmory et al., 2003) as well as to tumor models (Paglia et al., 1998; Lode et al., 2000; Niethammer et al., 2001; Xiang et al., 2000, 2001; Weth et al., 2001; Zoller and Christ, 2001). Furthermore, *Salmonella*-mediated oral DNA immunization was applied in a study for the treatment of the fungus *Penicillium marneffei* (Wong et al., 2002). Apart from the possibility to prevent infectious disease, *S. typhimurium*-mediated gene transfer has been also employed to complement monogenic defects (Montosi et al., 2000; Paglia et al., 2000) and to transfer therapeutic molecules (Urashima et al., 2000; Yuhua et al., 2001). The extraordinary versatility of *Salmonella* live vectors demonstrated here makes them a promising live DNA vaccine delivery system. Although, human clinical trials with DNA vaccines delivered by attenuated *Salmonella* have not yet been undertaken, it is expected that clinical trials with constructs carrying *P. falciparum*, HIV and measles DNA vaccines will be initiated soon (Levine et al., 2002).

Possible mechanism of *Salmonella*-mediated gene-transfer

The method of oral genetic vaccination with attenuated *Salmonella* as carrier is most likely based on the induction of adaptive immune responses as proposed by Darji et al. (1997, 2000). Orally administered *S. typhimurium aroA*⁻ reach the host via M cells of the Peyer's patches and are taken up by macrophages and dendritic cells (Hopkins et al., 2000) in the intraepithelial pocket where they reside in the phagocytic vacuoles (Fig.1.2; Fig. 1.3). These phagocytic cells are activated by the pathogen

and DCs might migrate probably into lymphnodes and spleen via the bloodstream or lymph fluid. During this time, the intracellular bacteria die after a view round of replication due to their attenuation and release the eukaryotic expression vector (Fig. 1.2).

S. typhimurium, unlike e.g. *Shigella*, remains in the phagosomal compartment of the host cell, where it releases the plasmid DNA after bacterial lysis. How the transfer of plasmid DNA into the cytoplasm of host cells is achieved is still unclear. Possibly, the bacteria encode a machinery to transfer macromolecules, particularly plasmid DNA, into target cells as it was shown for bacterial proteins injected into the cytosol by type III secretion systems in several Gram-negative bacteria (Galan and Collmer, 1999). In contrast, the transfer of DNA by bacterial ghosts has been demonstrated (Lubitz, 2001), rendering an active mechanism encoded by the microorganism highly unlikely. In addition, cell specificity has been observed for different bacterial species in their ability to transfer DNA from the phagosomal compartment. For example, *S. typhimurium* exclusively transfers DNA into primary murine and human macrophages and human dendritic cells *in vitro* (Darji et al., 1997; Montosi et al., 2000; Dietrich et al., 2001), whereas transfer into established cell lines of different origin was extremely inefficient (Darji et al., 1997; Grillot-Courvalin et al., 2002). Thus, it is more likely that particular host cell specific pathways exist that are exploited by the different bacterial species (Weiss, 2003).

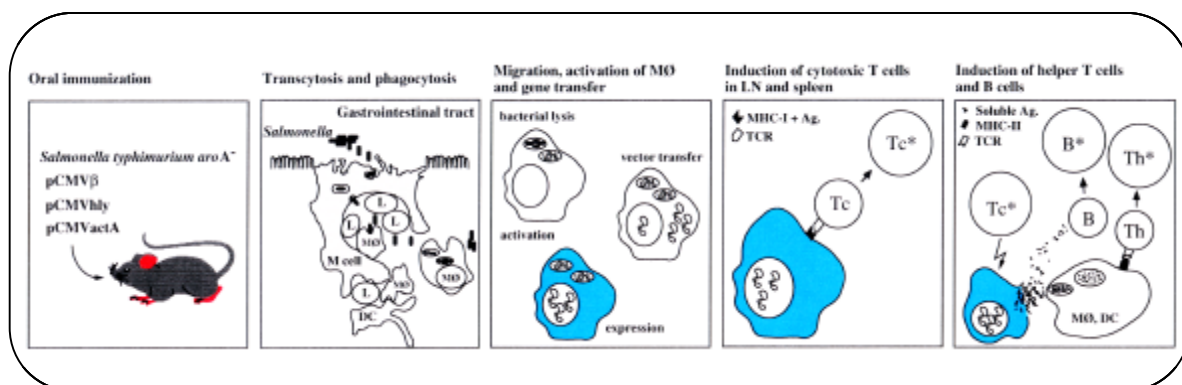


Fig.: 1.2: Schematic representation of *Salmonella*-mediated gene-transfer after oral immunization (Darji et al., 1997).

After transfer into the cytosol, the plasmid vector reaches the nucleus, where it is transcribed. This leads to the expression of non-secretory antigens in the cytosol of the host cell. In turn, specific cytotoxic T cells will be stimulated by the activated APCs (Fig. 1.2).

Upon infection of activated macrophages, *Salmonella* induce programmed cell death (Chen et al., 1996; Monack et al., 1996) through two different pathways. The immediate type is mainly induced by virulence factors of pathogenicity island I (Hersh et al., 1999) and is essential for the dissemination of *Salmonella* from the Peyer's patches to the deep organs (Monack et al., 2000). In comparison, the delayed cell death is primarily dependent on virulence factors of pathogenicity island II (Monack et al., 2001). This programmed cell death after infection by *Salmonella* shares features of apoptosis as well as of necrosis (Brennan and Cookson, 2000) and was described as pyroptosis by (Cookson and Brennan, 2001). Thus, intracellular antigens are possibly released after *Salmonella*-mediated oral DNA vaccination from infected macrophages and might be acquired by neighboring DCs, which could present the antigen via MHC class II to stimulate helper T cells as well as via MHC class I to activate cytotoxic T cells. Free antigen could also be responsible for the induction of antibody responses. In addition, it is possible that neighboring DCs take up the complete dying macrophages and re-present the antigens expressed by this cell via MHC class I and II via cross-presentation (Heath and Carbone, 2001) (Fig. 1.3).

Activation of CTL and helper T cells can occur not only locally in the Peyer's patches, but also in deep lymphoid organs, including mesenteric lymphnodes and spleen, after migration of antigen-presenting dendritic cells (Darji et al., 2000, unpublished; reviewed by Weiss, 2003). Another alternative for the induction of systemic immune responses after administration of plasmid carrying *Salmonella* was suggested by the recent finding that dendritic cells in the lamina propria can extend dendrites into the gut lumen to sample antigen (Rescigno et al., 2001). Thus, the recombinant *Salmonella* might be sampled by such DCs, which could in turn transport after *in vivo* transfection the antigen to the lymphoid organs and induce the immune responses.

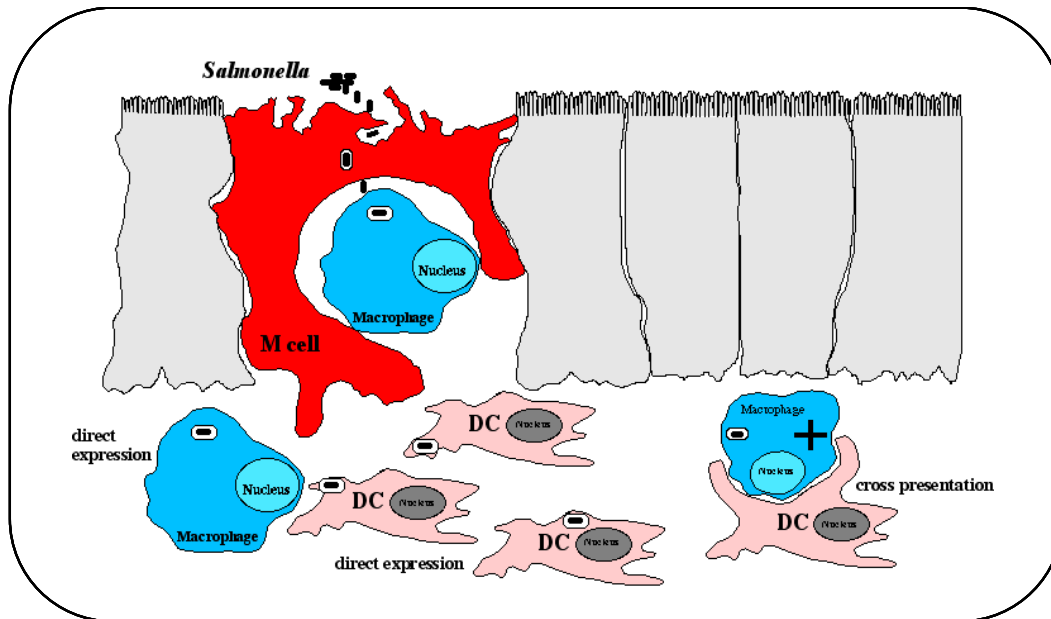


Fig. 1.3: Mechanisms of antigen presentation after *Salmonella*-mediated gene-transfer. Lysis of *Salmonella* after phagocytosis by APCs (macrophages and DCs) leads to transfer of the eukaryotic plasmid into the nucleus of the host cell where the antigen is transcribed leading to direct antigen expression in the cytosol. In addition, dendritic cells might take up dying macrophages and re-present antigens via cross-presentation.

Advantages of Salmonella-mediated DNA transfer

Favorable for this kind of vaccination is that the recombinant plasmids encoding the heterologous antigen are first constructed in lab strains of *E. coli* and can be introduced afterwards directly into *Salmonella* (e.g. by electroporation) without further manipulation, since both bacterial species are closely related.

Bacterial endotoxin and DNA sequence motifs can serve as adjuvants and enhance the immune response. The helper T cell response evoked by this kind of genetic immunization with *S. typhimurium aroA*⁻ seems to be strongly biased to a Th1-response as was expected for this specific bacterial carrier (Darji et al., 1997).

Salmonella-mediated mucosal DNA vaccination elicited cytotoxic and helper T cells as well as specific antibodies even after a single application and was far superior to

oral application with an equal amount of *Salmonella* expressing high amounts of heterologous antigen under control of a prokaryotic promoter (Darji et al., 1997). Additionally, only recombinant *Salmonella* carrying a eukaryotic expression plasmid could also induce T cell memory (Darji et al., 2000). Furthermore, *Salmonella* carrying an expression plasmid encoding listeriolysin O protected mice against a lethal challenge with *L. monocytogenes* (Darji et al., 1997).

However, despite this strong induction of T cells and specific IgG antibodies after mucosal administration, hardly any antigen specific IgA antibodies could be detected (Darji et al., 2000, unpublished). Moreover, the induction of an antibody response in general (including IgG) is only possible under optimal conditions (Darji et al., 2000). One problem might be that the *Salmonella aroA*⁻ strain, that is currently mainly used, is dying very quickly when containing high copy number plasmids (Garmory et al., 2002b) and thus only few cells might be transfected or only few plasmids might be transferred. Antigen expression also might be limited by an efficiently induced programmed cell death of the cells infected by these *Salmonella*. Although Urashima et al. (2000) could demonstrate many transgene expressing cells in the Peyer's Patches after *Salmonella*-mediated DNA transfer, such cells might not support efficient antibody production and/or the switch of B cells to IgA. Therefore, further improvements are required to generate strong local antibody responses in addition to the systemic response.

1.7 Optimization of bacterial DNA delivery systems

Depending on the nature of the invading pathogen, specific host responses are required for protection and clearance of these infectious agents. Therefore, selection of the most adequate strategy to stimulate the required immune response is essential for vaccine development, as mentioned before. In this respect, the exploitation of attenuated bacteria as antigen delivery system for DNA vaccines offers several tools to stimulate and modulate the appropriate type of immune response that is needed. The bacterial carrier and the antigen-coding plasmid are different entities. Modulation and specific targeting of the immune response should be possible by either choosing or engineering a proper bacterial carrier strain, alternatively by modifying the plasmid (e.g. by co-expression of cytokines or co-stimulatory molecules) itself.

1.7.1 Vector optimization

Manipulation of plasmid DNA vectors to achieve increased levels of expression *in vivo* is a laudable goal of DNA vaccine development. This approach is particularly attractive since changes of the vector sequence do not affect the formulation of the vaccine itself unlike other potential approaches that would involve additional components. Hence, they would increase the complexity of the vaccine (Ulmer et al., 1996).

Since it is generally believed that the level of expression *in vivo* obtained after DNA vaccination correlates with the immune response generated, there are several approaches to improve gene expression. These approaches include optimizing gene regulatory elements within the plasmid backbone or modifying the plasmid backbone itself to enhance gene expression. Thus, several possibilities exist to improve the plasmid vector:

Codon usage

Since codon bias has been detected in several species, the use of selective codons in a particular gene correlates with efficiency of gene expression (Lewin, 1994). For example, using an expression cassette for gp120 of HIV that was codon optimized for human vaccination already a single application induced a strong CD8⁺ T cell response in spleen and Peyer's patches compared to a non-codon optimized construct (Shata and Hone, 2001). Therefore, optimization of codon usage for eukaryotic cells enhanced antigen expression and subsequently led to enhanced CTL and protective immunity (Uchijima et al., 1998; Andre et al., 1998; Vinner et al., 1999).

Regulatory elements

Specific modifications to a vector that could increase overall protein expression include particular combination of promoter and terminator (Montgomery et al., 1993; Liang et al., 1996). Virally derived promoters, especially the CMV immediate early enhancer-promoter, provided the strongest gene expression *in vivo* compared to eukaryotic promoters (Manthorpe et al., 1993; Cheng et al., 1993). On the other hand, viral promoters can be down-regulated by IFN γ and TNF α , which might be induced upon immunization. This could lead to down-regulation of antigen expression

(Qin et al., 1997). Furthermore, the particular promoter chosen to drive expression of the recombinant antigen can influence the quality of immune responses (Medina et al., 2000).

The inclusion of enhancer elements such as intron A region of the cytomegalovirus promoter results in optimal gene expression due to splicing of the mRNA transcript (Chapman et al., 1991). Further, transcriptional termination elements (e.g. from the rabbit β -globin gene) that confer mRNA stability have been shown to enhance gene expression (Norman et al., 1997). Although it is in principle desirable to develop vectors for high level expression of protein, there may be also some mitigating factors. As examples, high levels of a protein that is toxic to cells could limit the duration of expression, and the expression of a protein that has effects on other cells (including cells of the immune system) could decrease immune responses directed against it (Ulmer et al., 1996).

Recently, strategies taking advantage of an alphaviral replicon to create an *in vivo* amplification system to improve DNA vaccine vectors were described (Tubulekas et al., 1997; Berglund et al., 1996). This type of construct can efficiently trigger cellular and humoral immune responses, although the alphaviral mediated expression is transient and lytic, which could be advantageous in terms of biosafety risks and tolerance-induction concerns (Berglund et al., 1998).

Plasmid stability

So far, the biggest problem seems to be the instability of the vector system. The use of high copy number plasmids most likely also influences the stability of the recombinant *Salmonella* in culture and *in vivo* (Garmory et al., 2002b). Usually recombinant high copy number plasmids carry antibiotic resistance markers to facilitate identification and to keep selective pressure in favor of plasmid retention. Such plasmids are unstable *in vitro* and *in vivo* in the absence of a stabilizing antibiotic, but can be stabilized *in vitro* by addition of the appropriate compound. Stability *in vivo*, however, is more critical and less certain. Therefore, different approaches have been suggested to address the issue of plasmid stability and to avoid the necessity for a stabilizing antibiotic. On the one hand, it was demonstrated that chromosomal integration of the heterologous antigen into *Salmonella* stabilizes antigen expression but also results in lower antigen concentrations (Hone et al., 1988; Strugnell et al., 1990). Studies by Cardenas & Clements revealed that the

intensity of immune responses against the B subunit of the heat-labile toxin (LTB) of enterotoxigenic *E. coli* after chromosomal expression were comparably weaker, although the construct was absolutely stable during the experiment (Cardenas and Clements, 1993). These data indicate that the amount of antigen that primes the gut-associated lymphoid tissue is very important for the development of an immune response against a foreign antigen and should be as high as possible.

Another possibility how stability of the expression construct can be reached, was demonstrated by the use of the *asd*⁺/ Δ *asd* balanced lethal system (Nakayama et al., 1988; Curtiss, III et al., 1990). Only *Salmonella* carrying a plasmid that complements the gene defect in the bacterium are able to survive. This combination of expression vector and mutant strain certainly eliminates the need for stabilizing antibiotics, but does not solve the problem of a general instable plasmid. The features of the plasmids and antigens that are expressed constitutively might diminish the vitality of the *Salmonella* carrier. The expression of heterologous antigens from *in vivo* inducible promoters was already shown to enhance stable expression and immunogenicity of a number of foreign genes by *S. typhimurium* (Chatfield et al., 1992a; Hohmann et al., 1995). Similarly, the maintenance of a plasmid by bacteria during growth can also be influenced by the copy number of the expression plasmid, its size and complexity or the expression level of the heterologous antigen. The relationship between plasmid instability and the copy number had been observed in previous studies (Coulson et al., 1994; Garmory et al., 2002, 2003). High copy number plasmids are rapidly lost from the bacteria, whereas a decrease in copy number reduces the level of metabolic burden and consequently improve plasmid retention in *Salmonella*. Since the copy number is usually regulated by the plasmid origin of replication (*ori*), expression plasmids carrying different low copy number *ori*'s should result in improved plasmid stability.

Targeting

The removal of elements not essential for eukaryotic expression, such as those contained in multi-purpose expression vectors, could also be advantageous for improved gene expression. A plasmid may also be engineered in the way that the encoded protein is either secreted or localized to the interior of the cell (Gurunathan et al., 2000). This might have dramatic effects of whether an antibody response is

elicited or not. Using N-terminal ubiquitination signals, which target the protein to proteosomes could enhance the delivery into the MHC class I pathway, thus inducing a stronger CTL response (Wu and Kipps, 1997).

CpG motifs

In addition, the modification of vectors could inadvertently introduce specific nucleotide sequences (e.g. CpG-motifs) that have modulatory effects on immune cells and can act as adjuvant by stimulation of these cells via Toll-like receptor 9 (TLR 9) to contribute to the immunogenicity of DNA vaccines. GC-rich DNA from bacterial sources was shown to stimulate natural killer (NK) cells (Yamamoto et al., 1992), B lymphocyte activity resulting in antibody production (Messina et al., 1991; Krieg et al., 1995b) and can directly induce professional APCs to secrete cytokines such as IFN γ , IL-12, IL-6, IL-18 and TNF α (Klinman et al., 1996; Stacey et al., 1996; Jakob et al., 1998). Likewise, CpG oligodeoxynucleotides can stimulate primary T cells in the absence of antigen-presenting cells (Bendigs et al., 1999). After substitution of a kanR-selectable marker for a CpG-containing ampR gene in a β -galactosidase-encoding plasmid, the altered plasmid induced higher IgG antibody, CTL and IFN γ production (Sato et al., 1996). The same effect was observed, when additional CpG motifs were introduced into the plasmid backbone, suggesting that the innate immune response stimulated by CpG motifs could promote antigen-specific humoral and cell mediated immunity (Klinman et al., 1997, 1999; Roman et al., 1997; Krieg et al., 1998). The use of additional CpG motifs may decrease the amount of vaccine required to induce an immune response rather than increase the absolute magnitude of a response (Gurunathan et al., 2000).

Expression of immunostimulatory DNA adjuvants, such as open reading frames for cytokines and costimulatory molecules, may also be useful to enhance or modulate immune responses induced by DNA vaccines *in vivo* (Ulmer et al., 1996; Gurunathan et al., 2000). Recent work by Krieg et al. (1998) demonstrated that eliminating suppressive motifs (tandem repeats of GpC) from the plasmid backbone of a DNA vaccine improved its immunogenicity. These observations display the complex interaction between DNA sequence motifs and the immune system. In contrast, the CpG motifs that induce optimal stimulation in mice (see above) are less effective on

cells of primate origin and sequence motifs optimally active in humans will have to be introduced when such types of vaccines enter the clinic.

Cytokines and Costimulatory molecules

Cytokines and costimulatory cell surface molecules play a crucial role in generation of effector T-cell subsets. Hence, several groups have exploited these molecules, encoded by plasmid DNA, to enhance or bias the immune response generated by DNA vaccination (reviewed by Gurunathan et al., 2000). Yuhua et al. (2001) demonstrated that oral cytokine gene therapy with IL-12 and GM-CSF (granulocyte-macrophage colony-stimulating factor) using live attenuated *Salmonella* significantly protects mice against two unrelated tumors. A dual-function DNA vaccine encoding a carcinoembryonic antigen fused with the costimulatory CD40 ligand trimer induced T cell-mediated protection against murine colon carcinoma (Xiang et al., 2001). Furthermore, the delivery of plasmid encoded cytokines by live attenuated *Salmonella* was able to modulate the immune responses in a variety of experimental models, i.e. influencing cytokine and antibody production to bystander co-administered heterologous antigens, modifying the immune response during a parasitic infection, and extending the life expectancy in melanoma-bearing mice (Rosenkranz et al., 2003).

Multivalent vaccines

Expression of two or multiple genes in the same cell, bicistronic or multicistronic vectors with internal ribosomal entry site (IRES) could be particularly useful in constructing multivalent vaccines from two or more different antigens from the same or different pathogens (Wild et al., 1998) or an antigen in combination with a cytokine or costimulatory molecule.

Moreover, co-delivery of two compatible eukaryotic expression plasmids encoding either the glycoprotein D gene of pseudorabies virus (PrV gD) or prothymosin as adjuvant together in the same *Salmonella choleraesuis* has been demonstrated to confer protective immunity against pseudorabies and enhance the vaccine efficacy compared to the application of *Salmonella* carrying the PrV gD expression plasmid alone (Shiau et al., 2001). Thus, the use of compatible plasmids in the same bacterium is likely to provide the possibility of delivering more than one antigen either

from the same or of different pathogens or to coadminister cytokines or costimulatory molecules together with the antigen.

Thus, optimization might be possible at various properties of the expression plasmid and a systematic approach is required to find out the optimal combination for a particular vaccine to elicit appropriate protective responses.

1.7.2 Optimization of the bacterial carrier

Many of the bacteria that target inductive sites and cells of the immune system display regional tropisms often manifested as colonization of specific mucosal surfaces or internal organs. The class of immune response that is evoked depends not only on the general features of the bacterial carrier, but also on the nature of the specific mutations affecting its virulence properties (Dunstan et al., 1998; Medina and Guzman, 2001). Depending on the attenuating mutations, strains may differ in their capacity to elicit antibody or cell-mediated immune responses (VanCott et al., 1998; Medina et al., 1999). For example, *S. typhimurium aroA*⁻ mutants elicit strong specific antibody and T helper cell responses, since Th1 cells and IFN γ are required for clearance of the *aroA*⁻ strain. In comparison, a *PhoP*⁻ null phenotype promotes potent innate immune responses of macrophages by involving CD14 and TLR4 as well as iNOS/O₂⁻. Thus innate responses are sufficient for host defense (VanCott et al., 1998).

This demonstrates that the choice of the attenuated *Salmonella* strain is critical. The class of immune response elicited against the heterologous antigen is influenced by the dominant type of immunity promoted by the carrier itself. Therefore, similar to testing properties of the plasmid, systematic testing of variant *Salmonella* strains with regard to immunomodulating properties is essential for the development of improved oral vaccines.

1.8 Aims of this work

Within the framework of this thesis, the vector system currently used for *Salmonella*-mediated gene transfer should be improved, on the one hand, by the use of different eukaryotic promoters and, on the other hand, on the basis of increased plasmid stability in *Salmonella*.

At present, all studies in this laboratory were performed using the commercially available high copy number plasmid pCMV β encoding the model antigen β -galactosidase or substituted by other relevant antigens like listeriolysin from *L. monocytogenes* or LACK (Leishmania homologue of receptors for activated C kinase) from *Leishmania major*. Although these constructs could stimulate a strong cytotoxic as well as helper T cell response, the induction of an antibody response is only possible under optimal conditions and is primarily systemic and not mucosal as might have been expected for a mucosally applied vaccine (Darji et al., 1997, 2000). Although the cytomegalovirus immediate early promoter and enhancer (CMV promoter) is a strong promotor for antigen-expression, several groups have shown that strong, constitutive, viral promoters do not cause long-term transgene expression *in vivo* (Scharfmann et al., 1994; Challita and Kohn, 1994; Rettinger et al., 1994). Moreover, transgene expression from viral promoters is inhibited by cytokines like IFN γ and TNF α (Qin et al., 1997). These observations might interfere with the ability of recombinant *S. typhimurium aroA*⁻ to induce local antibodies and T cell memory after only a single administration, since *aroA*⁻ mutants especially stimulate IFN γ -secretion for host defense. To circumvent this problem, different eukaryotic promoters should be constructed. Their potential to drive antigen expression after oral transgene immunization with *S. typhimurium aroA*⁻ and subsequently to generate specific immune responses should be compared to the original pCMV β plasmid. Therefore, the CMV promoter in the eukaryotic expression vector pCMV β was substituted by the human lysozyme promoter (Dighe et al., 1995; Clarke et al., 1996), the human elongation factor 1 alpha (Uetsuki et al., 1989; Kim et al., 1990), the ubiquitin promoter (Nenoi et al., 1996) or the CD11c promoter (Lopez-Cabrera et al., 1993). In addition, the human interferon regulatory factor 1 (IRF-1) gene promoter (Sims et al., 1993) was also included, which is activated by IFN γ . Hence, the IRF-1 promoter should be up-regulated by IFN γ that is produced upon immunization for host defense against *S. typhimurium aroA*⁻.

From this first set of experiments, it became obvious that first of all the bacterial delivery system had to be optimized, since all recombinant strains rapidly lost their plasmids in culture.

One basic problem was that the currently used *S. typhimurium aroA*⁻ strain is dying very quickly when harboring high copy number plasmids (Garmory et al., 2002).

Thus, only few cells might be transfected or only few plasmids are transferred resulting in limited antigen expression and therefore an insufficient stimulation of humoral responses. Along this line, another problem appears to be the stability of the vector system, since the copy numbers of the plasmids that are employed so far influence the stability of the recombinant bacteria in culture as well as *in vivo* (Coulson et al., 1994). A decrease in copy number should reduce the level of metabolic burden, thus plasmid retention in *Salmonella* should be obtained with plasmids of low copy number.

The copy number is usually regulated by the plasmid origin of replication (ori). Therefore, new expression plasmids on the background of the pCMV β vector were designed containing different low copy number ori's. Transformation of *Salmonella* with such vectors should result in improved plasmid stability and possibly an increased level of antigen expression and immune responses.

The new plasmids were designed to consist of cassettes that could be easily exchanged when required. In the following, the pUCori of pCMV β was exchanged against the low copy number ori's pMB1, p15A and the very low copy number ori pSC101. These newly constructed vectors were compared with the conventionally used pCMV β with regard of plasmid stability *in vitro* in the absence of a stabilizing antibiotic as well as *in vivo*. Using these strains, induction of cellular and humoral immune responses against the model antigen β -galactosidase should be analyzed and compared after single or multiple administrations of these recombinant *Salmonella* strains. In addition, the capacity of these strains to protect mice against a lethal dose of *Listeria monocytogenes* should be examined using listeriolysin (LLO) as antigen. These protection studies are particular important, because single administration of the original recombinant *Salmonella* only partially protected mice (Darji et al., 1997, 2000).

Since the different ori's should be able to coexist in one and the same bacterium *S. typhimurium aroA*⁻ should be transformed with two plasmids containing compatible origins of replication and plasmid retention in *Salmonella in vitro* as well as *in vivo* should be investigated. To facilitate the identification of both plasmids, the ampicillin resistance marker (AmpR) should be replaced by a tetracycline resistance marker (TetR). Using β -galactosidase and LLO as antigens in the two compatible plasmids, immune responses, that will be evoked after vaccination against both antigens simultaneously, should be investigated.

The opportunity to deliver two compatible plasmids at once within the same bacterium should offer a simple and versatile tool to enhance and/or modulate the immune responses by co-delivering additional antigen, cytokines or costimulatory molecules. Thus, a step towards a second generation of *Salmonella*-based oral DNA vaccine should be taken with these new strains.

2 Materials

2.1 Bacteria

2.1.1 *Escherichia coli* strains

The *E. coli* strain TOP 10 (Invitrogen, Groningen, The Netherlands) was used for general cloning of plasmids.

2.1.2 *Salmonella typhimurium* strains

The auxotrophic, non-virulent *S. typhimurium aroA*⁻ strain SL7207 (*S. typhimurium* 2337-65 deriv., hisG46, DEL407 [aroA::Tn10 {Tc-s}]; kindly provided by Dr. B. A. D. Stocker, Stanford, USA) was used as carrier for the *in vitro* assays as well as for the *in vivo* studies in the mouse model.

2.1.3 *Listeria monocytogenes* strains

The wild-type strain *L. monocytogenes* EGDe, serotype 1/2a (Leimeister-Wächter and Chakraborty, 1989) was used for *in vivo* protection assays.

2.2 Cell lines

BHK-21: fibroblast-like cell line; generated from kidney cells of asexual hamsters (BHK = baby hamster kidney)

HEp-2: adherent, epithelial HeLa contaminant, derived from human (*Homo sapiens*), ATCC CCL-23.

2.2.1 Mouse strains

6-to 8-week-old female BALB/c (H-2^d) mice were used. Mice were maintained from Harlan Winkelmann (Borken, Germany).

2.3 Culture Media

2.3.1 Culture media for bacteria

2.3.1.1 LB-Broth

Luria Bertani broth (LB, Sambrook et al., 1989) was utilized for culture of *E. coli* und *S. typhimurium aroA⁻* strains in LB-broth or on LB-agar plates. For *in vivo* studies, *S. typhimurium aroA⁻* was grown in LB-broth supplemented with 1.5% final NaCl. Recombinant bacteria were cultivated by addition of either 100 µg/ml ampicillin or 25 µg/ml tetracycline or a combination, where required.

LB-broth: 10 g bacto-trypton; 5 g yeast extract; 10 g NaCl;
H₂O ad 1000 ml

Agar plates: For production of agar plates, LB-broth was supplemented with 15 g/l agar.

Ampicillin stock solution : 100 mg/ml ampicillin sodium salt was dissolved in H₂O.

Tetracycline stock solution: 25 mg/ml tetracycline was dissolved in 60% EtOH (v/v in H₂O).

Streptomycin stock-solution: 30 mg/ml streptomycin was suspended in H₂O.

All antibiotics were stored in aliquots at -20°C and were added just before use, i.e. for agar plates right before pouring the plates at 50°C.

2.3.1.2 SOC-medium

SOC-medium (Sambrook et al., 1989) was used for culture of recombinant bacteria directly after their transformation.

SOC-medium : 100 ml SOB-medium compl.; 10 mM MgCl₂; 10 mM MgSO₄; 20 mM glucose

SOB-medium compl. : 20 g bacto-trypton; 5 g bacto-yeast extract; 0.5 g NaCl; H₂O ad 1000 ml; autoclaved for sterilization, 20 ml 1M MgSO₄ were added before use

2.3.1.3 BHI-broth

Brain Heart Infusion (BHI)-broth or BHI-agar plates (Difco, Detroit, MI) were used for culture of *L. monocytogenes*.

2.3.2 Medium for cell culture

2.3.2.1 IMDM complete

Iscove's modified Dulbecco's modified Eagle's medium (IMDM-medium, Gibco BRL, Eggenstein, Germany) is a complete powdered medium and was prepared according to manufacturer's protocol. After addition of 10% FCS, 2 mM L-glutamin, 0.25 µM β-mercaptoethanol (Serva, Heidelberg, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (Cytogen, Ober-Mörlen, Germany), IMDM complete medium was utilized as standard medium for tissue culture.

FCS : Integro, Zaandam, The Netherlands; were heated up for 30 min at 65°C to inactivate the complement system

2.4 Oligonucleotides

All primers were ordered from GIBCO BRL. Nucleotides are listed in 5'-3'-direction.

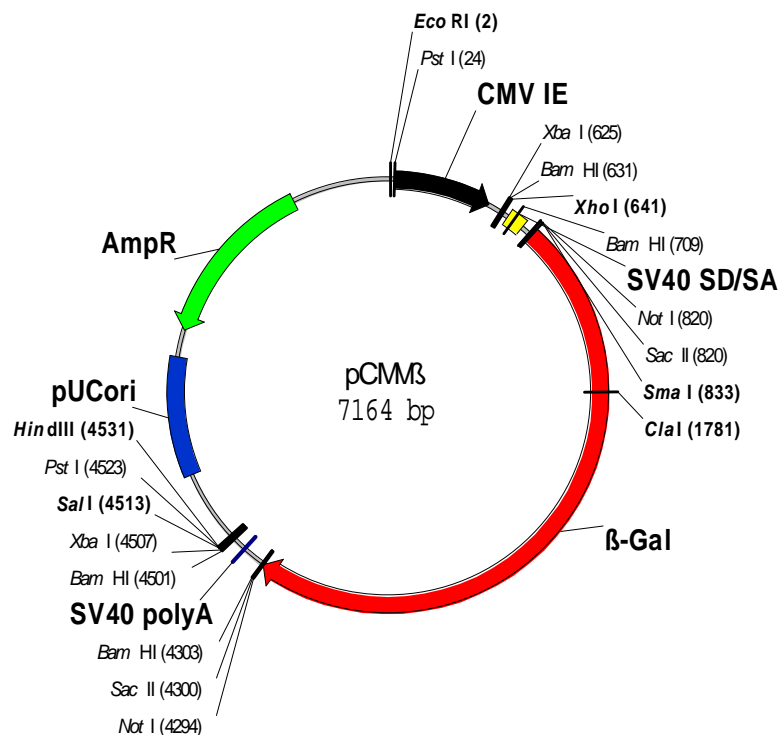
- 1 AmpR VORW EcoRI: GCGCGC^{*}GAATTC TTCAAATATGTATCCGCTCATGAG
- 2 AmpR REV BKS: AGATCTGGTACCACTAGTTTACCAATGCTTAATCAGTGA
- 3 pUCori VORW Sall: ATAAGAGTCGACAGGAACCGTAAAAAGGCCGCGTTG
- 4 pUCori REV SKB: ACTAGTGGTACCACTAGTTTACCAATGCTTAATCAGTGA
- 5 PTetR Vorw EcoRI: ATAAGAGGAATTC TCATGTTTGACAGCTTATCATCGATAA
- 6 TetR Rev SpeI: GCGCGCACTAGTTCAGGTCGAGGTGGCCCGGCT
- 7 pMB1 Vorw Sall: ATAAGAGTCGACTGGCATTGACCCTGAGTGATTTTTCTC
- 8 p15Aori Vorw Sall: GCGCGCGTCGACTCAGCGCTAGCGGAGTGTATA
- 9 p15ori Rev BglII: ATAAGAGAATCTTTAATAAGATGATCTTCTTGAGATCGTTTT
- 10 pWSK29 Vorw Sall: GCGCGCGTCGACGCAGACCCGCCATAAACGCCCTG
- 11 pSC101 Rev BglII: ATAAGAGAATCTTCAGATCCTTCCGTATTTAGCCAGTAT
- 12 PCmR Vorw PstI: ATAAGACTGCAGTACCTGTGACGGAAGATCACTTCGCAG
- 13 CmR Rev SpeI: GCGCGCACTAGTTTACGCCCCGCCCTGCCACTCATC
- 14 Seq1 pCMVβm: GACCTGCAGGCATGCAAGCTC
- 15 Seq2 pCMVβm: AGATCTGGTACCACTAGT
- 16 Seq3 pCMVβm: ACTAGTGGTACCACTAGT
- 17 Seq4 pCMVβm: GGATCCTCTAGAGTCGAC

* restriction sites are indicated in color

2.5 Plasmids

2.5.1 pCMV β

The eukaryotic expression plasmid pCMV β (Clontech, Palo Alto, CA) is a reporter plasmid for the expression of β -galactosidase in mammalian cells driven by the human cytomegalovirus immediate early promoter (P CMV IE). It contains a splice donor/splice acceptor site (SV40 SD/SA) and polyadenylation signal from SV40 (SV40 polyA) as well as the full-length *E. coli* gene *lacZ* with eukaryotic translation initiation signals for expression of β -galactosidase (β -Gal). Further, it includes an ampicillin resistance marker (AmpR) and the origin of replication from pUC (pUCori).

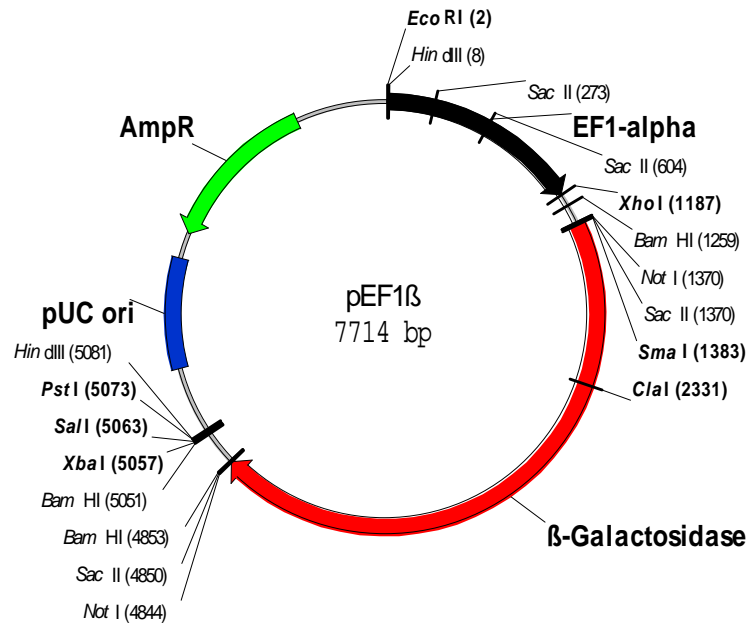


2.5.2 pEF1 β

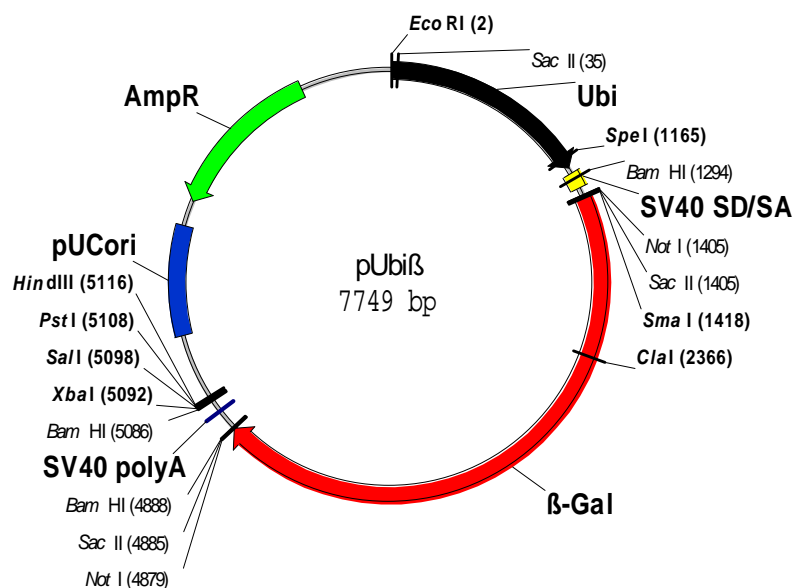
Since it was reported that the CMV IE promoter could be down-regulated by IFN γ (Qin et al., 1997), which is strongly induced after immunization with *S. typhimurium*

aroA⁻ for host defense, a series of plasmids was constructed that contain different kind of promoters to avoid this problem.

pEF1 β is a plasmid for eukaryotic expression of β -galactosidase under the human elongation factor 1 α promoter (Uetsuki et al., 1989; Kim et al., 1990) on the backbone of pCMV β (Garbe, A.I., Braunschweig).



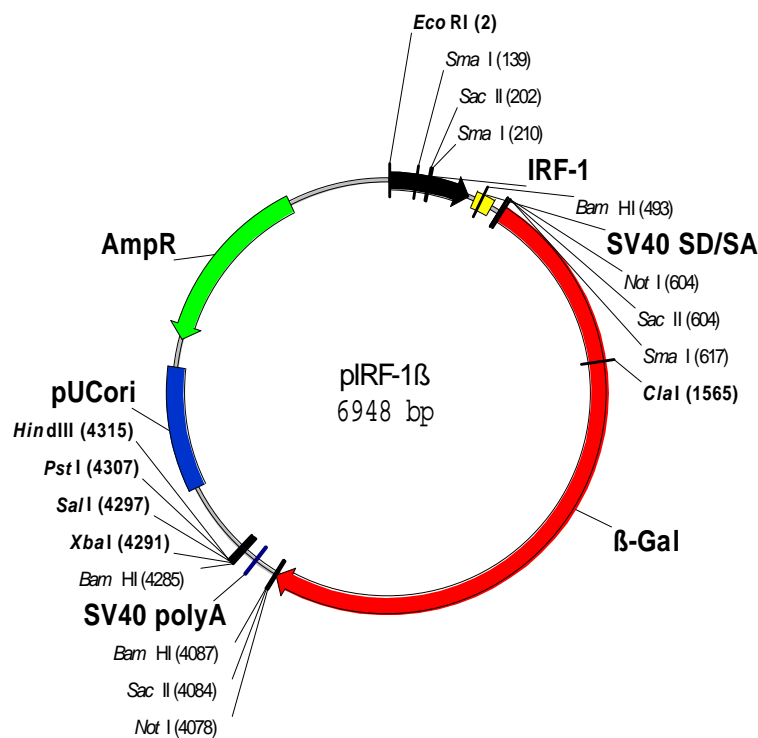
2.5.3 pUbi β



Expression plasmid for β -galactosidase driven by the human ubiquitin promoter (Nenoi et al., 1996) on the backbone of pCMV β (Garbe, A.I., Braunschweig).

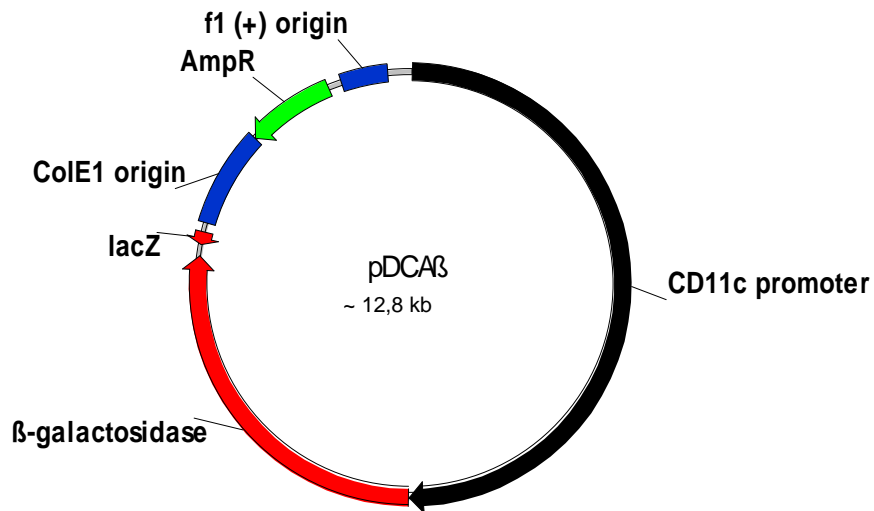
2.5.4 pIRF-1 β

Plasmid for eukaryotic expression of β -galactosidase driven by the human interferon regulatory factor-1 (IRF-1) promoter (Sims et al., 1993) on the backbone of pCMV β (Garbe, A.I., Braunschweig). The IRF-1 promoter should be up-regulated by IFN γ that is induced due to immunoreactions of the host.



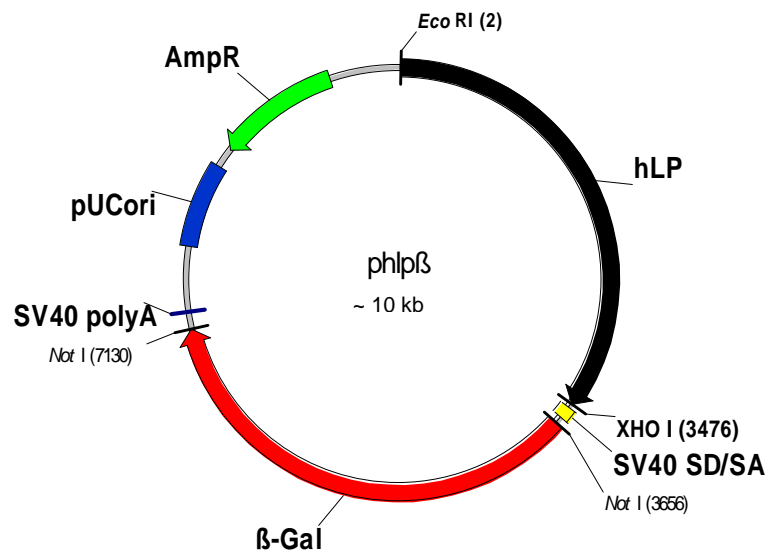
2.5.5 pDCA β

Expression plasmid for β -galactosidase (Garbe, A.I., Braunschweig) under the CD11c- β -globin promoter (CD11c promoter) on the backbone of pBSCD11c (kindly provided by T. Brocker).



2.5.6 pHLP β

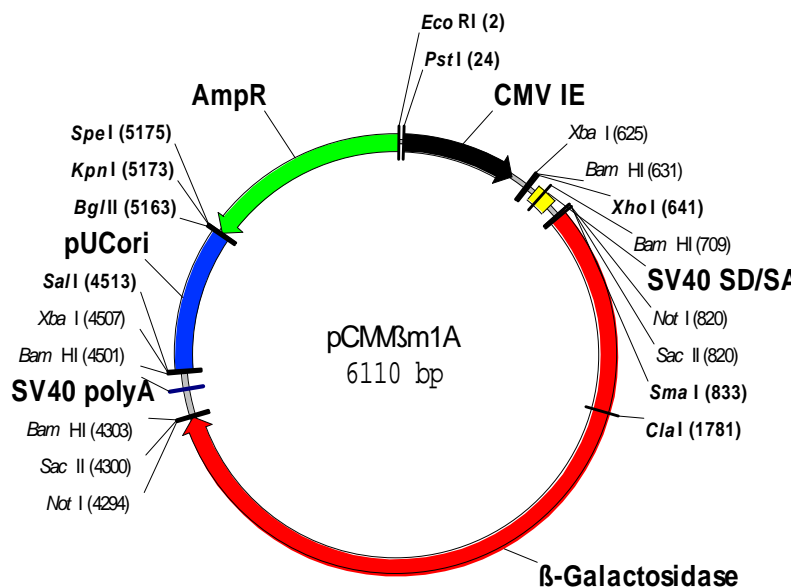
Expression plasmid for β -galactosidase driven by the human lysozyme promoter (hLP) (Dighe et al., 1995; Clarke et al., 1996) on the backbone of pCMV β (Garbe, A.I., Braunschweig).



2.5.7 pCMV β m1A

For the cloning of the eukaryotic expression plasmids with different origins of replication and the possibility to exchange the antibiotic resistance, the eukaryotic expression plasmid pCMV β (Clontech, Palo Alto, CA) was reconstructed to introduce unique restriction enzyme sites resulting in pCMV β m1A.

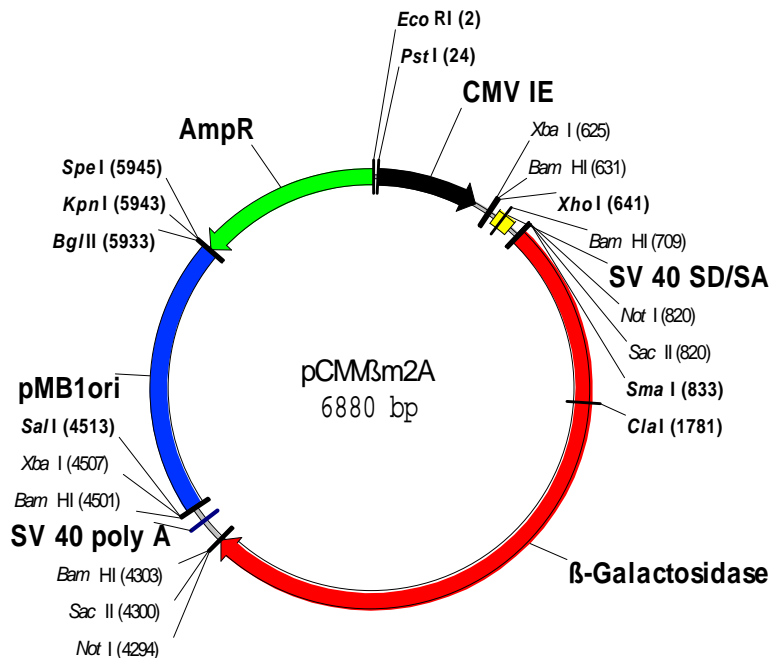
The pCMV β was digested with *Eco*RI and *Sal*I to remove the pUCori and the ampicillin resistance gene. The pUCori was then amplified by PCR using primers pUCori VORW *Sal*I and pUCori REV SKB providing restriction enzyme sites *Sal*I 5' and *Spe*I, *Kpn*I and *Bgl*II 3' of the ori. For the ampicillin resistance gene the primers AmpR VORW *Eco*RI and AmpR REV BKS were used to introduce 5' *Bgl*II, *Kpn*I and *Spe*I and 3' *Eco*RI. Both overlaying fragments were joined together by amplification in a second PCR. Finally, the fragment pUCori-AmpR was ligated via *Eco*RI and *Sal*I with the pCMV β fragment carrying the lacZ gene under the control of the CMV promotor to give pCMV β m1A.



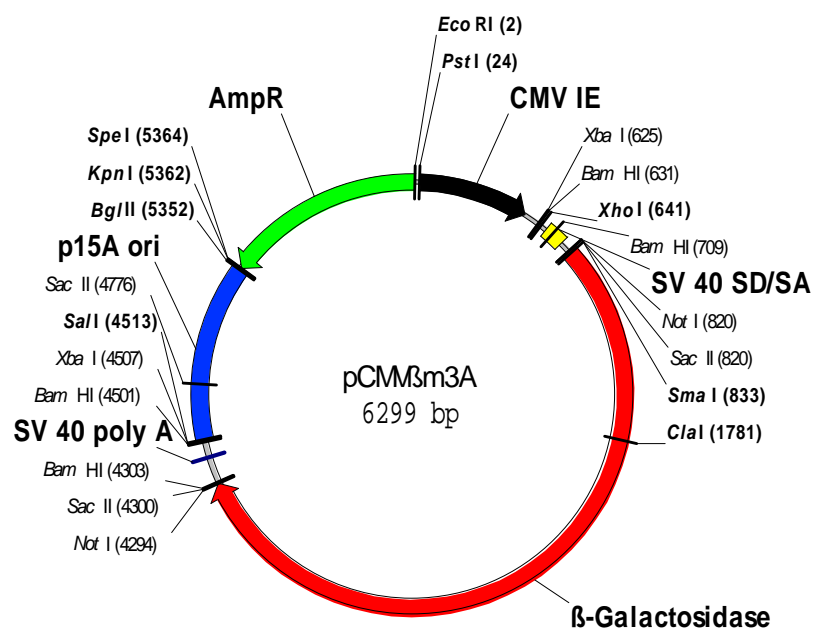
2.5.8 pCMV β m2A

The low copy number pMB1replicon from pBR322 (Sutcliffe, J.G., 1979), containing the pUCori and the rop gene from the ColE1 derivative pMB1, was amplified by PCR

with the primers pMB1 Vorw SalI and pUCori REV SKB. After digestion with BglII/SalI, the pMB1 replicon was inserted in pCMV β m1A, creating pCMV β m2A.



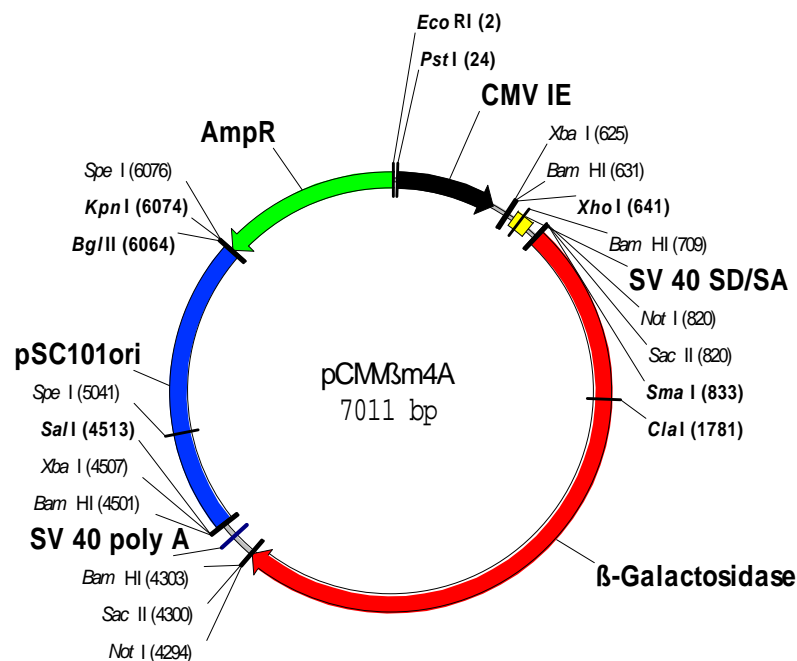
2.5.9 pCMV β m3A



pCMV β m3A was constructed by cloning the PCR-amplified low copy number p15Aori from pACYC 184 (Chang and Cohen, 1978) with primers p15Aori Vorw Sall and p15Aori Rev BglII in pCMV β m1A. pACYC184 is a low copy number cloning vector that carries the ori from p15A. Plasmids carrying this origin of replication can co-exist with vectors carrying the ColE1 origin such as pBR322 or pUC19.

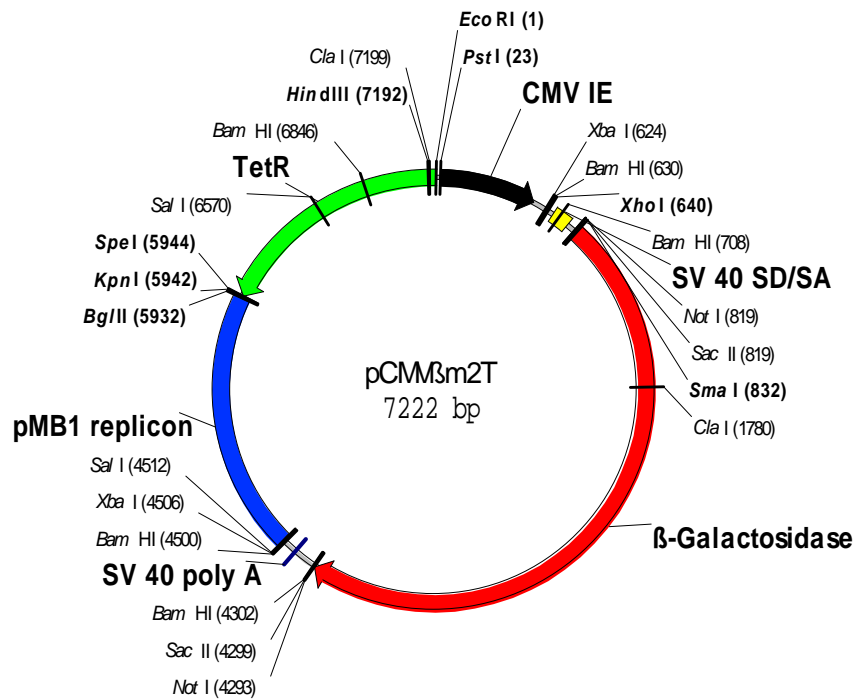
2.5.10 pCMVm4A

For construction of plasmid pCMV β m4A, PCR amplification of the very low copy number pSC101ori was performed using pWSK29 (Wang and Kushner, 1991) as a template with pWSK29 Vorw Sall and pSC101 Rev BglII and cloned into pCMV β m1A after digestion with BglII/Sall.



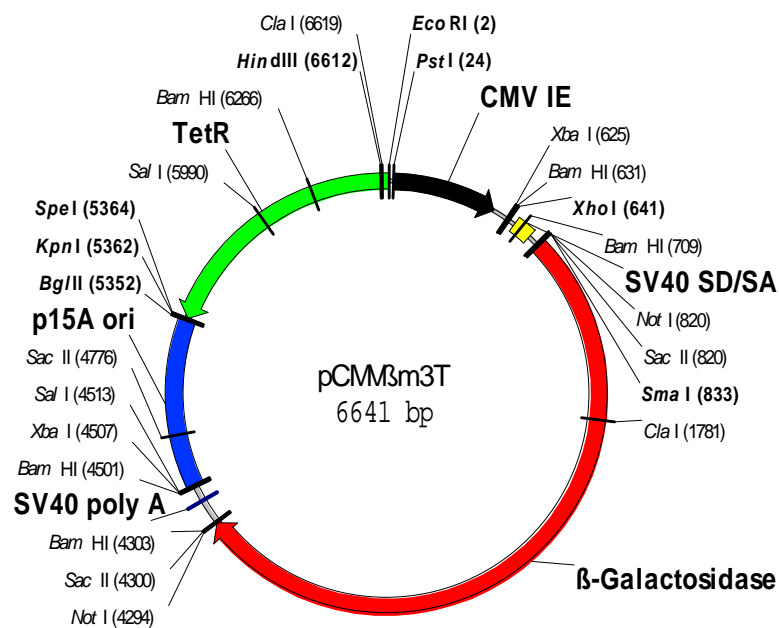
2.5.11 pCMVm2T

To insert the tetracycline resistance gene instead of the ampicillin cassette in pCMV β m2T the TetR gene was PCR amplified with PTetR Vorw EcoRI and TetR Rev SpeI and ligated via EcoRI/SpeI into pCMV β m2A that removed the ampicillin resistance at the same time.



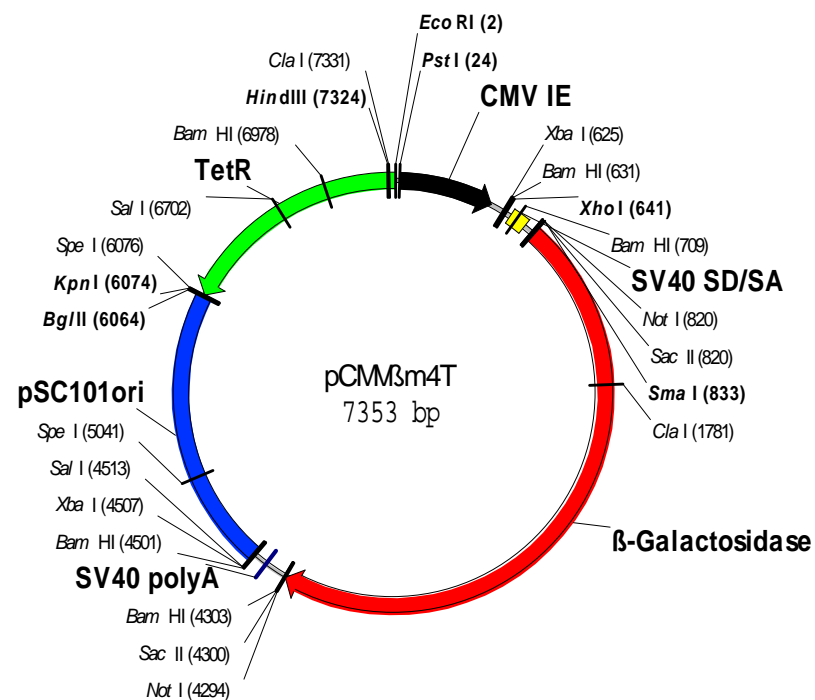
2.5.12 pCMVβm3T

For cloning of pCMVβm3T, the amplified TetR gene (see 2.5.11) was introduced into pCMVβm3A via *Eco*RI and *Spe*I similar to pCMVβm2T.

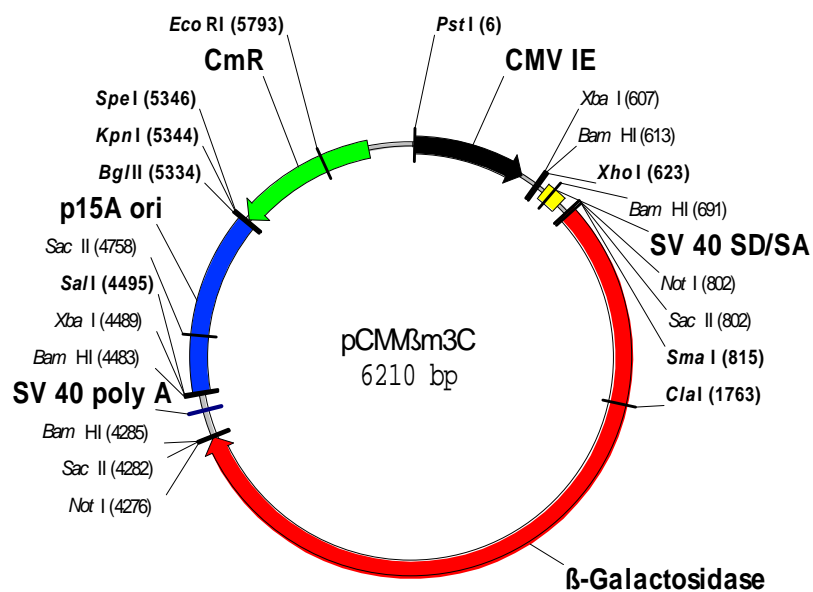


2.5.13 pCMV β m4T

pCMV β m4T was constructed by exchange of AmpR in pCMV β m4A against TetR from pCMV β m2T after digestion with EcoRI and BglII.



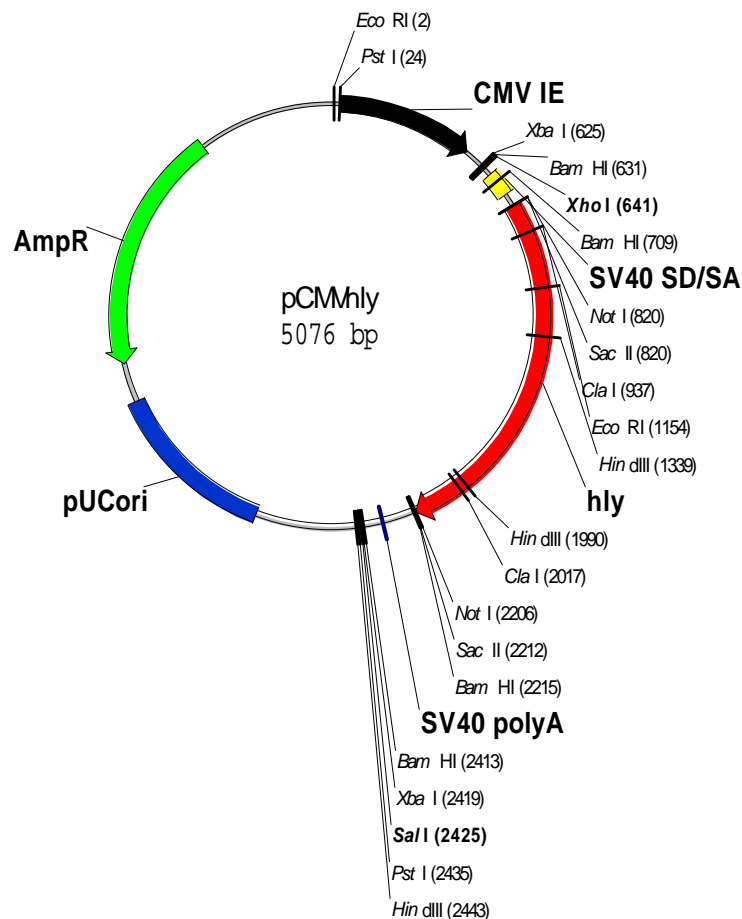
2.5.14 pCMV β m3C



The chloramphenicol resistance gene (CmR) was amplified from plasmid pACYC184 by PCR using the primers PCmR Vorw PstI and CmR Rev SpeI and was introduced into pCMV β m3A by digestion and ligation with the restriction endonucleases PstI and SpeI.

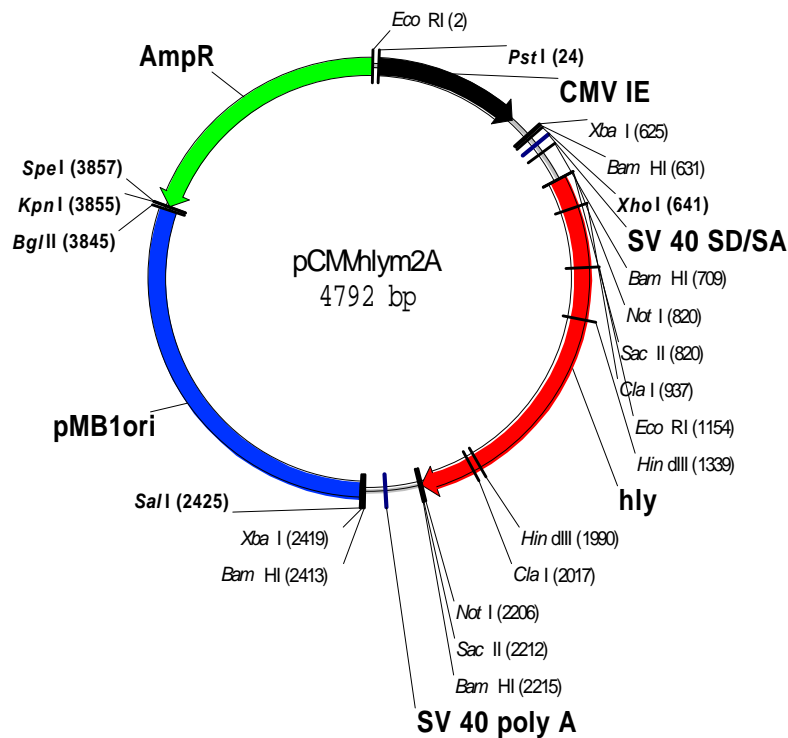
2.5.15 pCMVhly

The plasmid pCMVhly (Darji et al., 1997) contains a fragment of the hly gene from *L. monocytogenes* encoding a non-hemolytic variant of listeriolysin as antigen. This plasmid was used for protection studies against infection with *L. monocytogenes*.



2.5.16 pCMVhlym2A

The hly gene was excised from pCMVhly with NotI and inserted into pCMV β m2A by replacing lacZ, thus giving pCMVhlym2A. pCMVhlym2A was used as a low copy number vector in protection studies.



2.6 Antibodies

- anti- β -galactosidase : monoclonal IgG2a; purified from ascites of a mouse hybridoma; recognizes E. coli β -galactosidase; Conc.: 2 mg/ml (Promega, Mannheim, Germany)
- goat-anti-mouse IgG (h+l) : polyclonal; peroxidase conjugated; conc.: 0,8 mg/ml (Dianova, Hamburg, Germany)
- rat-anti-mouse IFN γ : monoclonal IgG1; clone R4-6A2; conc.: 1 mg/ml (Pharmingen, San Diego, CA)
- rat-anti-mouse IFN γ : monoclonal IgG1; clone XMG1.2; biotinylated; conc.: 0.5 mg/ml (Pharmingen, San Diego, CA)

2.7 Antigens

2.7.1 β -Galactosidase

The β -galactosidase protein (Boehringer, Mannheim, Germany) was used as antigen in cellular assays and ELISA. The synthetic peptide aa 877-885: TPHPARIGL (H-2k^d) was utilized for BALB/c mice in ELISPOT and the JAM-assay.

2.7.2 Listeriolysin

Purified, hemolytically active listeriolysin (LLO) (Darji et al., 1995) was used in proliferation assays. The LLO peptide aa 91-99: GYKDGNEYI (H-2k^d) (Pamer et al., 1991) was used for ELISPOT.

3 Methods

3.1 Molecular biological methods

3.1.1 Agarose gel electrophoresis

The agarose gel electrophoresis was applied for separation of linearized, double-stranded DNA fragments. The separation depends on the migration of DNA fragments in an electric field due to their molecular size. Because of that the size of the DNA fragments can be estimated by comparison with marker DNA comprising molecules of known size. This method was employed for analytical as well as for preparative purposes. In this study, gels were used at concentrations of 0.8 % (w/v) agarose (Appligene, Heidelberg, Germany) in TAE buffer and run at 120-140 V. DNA fragments became visible with UV light after staining with ethidium bromide and were documented photographically after sufficient separation.

TAE-buffer : according to Sambrook et al., 1989

Marker-DNA : mix of HindIII-digested λ -DNA and HaeIII-digested p Φ X174-DNA (MBI-Fermentas, St. Leon-Rot, Germany)

Electrophoresis-system: Horizon 11.14 (Gibco BRL Eggenstein, Germany)

3.1.1.1 Isolation of DNA from agarose gels

The DNA fragment of interest was carefully cut out from the gel. Subsequently, the DNA was extracted from the gel using the „QIAquick Gel Extraction Kit“ (Qiagen, Hilden, Germany) according to manufacturer's protocols. DNA was always eluted in 50 μ l water and stored at -20°C until use.

3.1.2 Isolation of Plasmid-DNA

Depending on the required amount of plasmid DNA, mini or maxi plasmid preparations were performed to isolate DNA from bacteria. To purify plasmid DNA from *E. coli* strains, the “GFX Micro Plasmid Prep Kit” (Amersham Biosciences, Freiburg, Germany) was used for analytical and the “Qiagen Plasmid Maxi Kit” (Qiagen, Hilden, Germany) for preparative purposes. The “Qiagen Plasmid Mini Kit” (Qiagen, Hilden, Germany) was employed for purification of plasmid DNA from *Salmonella* strains.

3.1.3 Determination of DNA concentration

3.1.3.1 Optical determination

For optical determination of the DNA concentration, the extinction of a sample was measured at 260 nm. The OD₂₆₀ of 1 corresponds (at a diameter of the cuvette of 1 cm) to a DNA concentration of 50 µg/ml.

Photometer : BioPhotometer (Eppendorf, Hamburg, Germany)

3.1.3.2 Visual comparison with marker DNA

This method only permits the determination of concentration from linearized DNA. Thereby, DNA fragments can be quantitated by running a sample alongside marker DNA that contains defined quantities and sizes of DNA fragments. The amount of sample DNA loaded can be estimated by visual comparison of the band intensity with that of the marker DNA.

3.1.4 Molecular Cloning

Molecular cloning comprises the cleavage of plasmid DNA with restriction enzymes and joining to foreign DNA fragments by the use of ligase. Thereby, fragments of foreign DNA and vector DNA carrying noncomplementary protruding termini, identical

protruding or blunt-ended termini can be covalently linked. The resulting recombinant plasmids are then used to transform bacteria.

3.1.4.1 Digestion of DNA with restriction enzymes

Plasmids and PCR products were digested with appropriate restriction endonucleases for analysis and preparation of defined linear DNA strands. Digestion of DNA was carried out using optimal buffer and temperature conditions for each enzyme according to manufacturer's protocol. Finally, an aliquot of the digestion mix was analyzed by agarose gel electrophoresis for completion of digestion.

3.1.4.2 Ligation of DNA

Ligation of a foreign DNA fragment to a linearized plasmid vector involves the formation of phosphodiester bonds. In this study, T4 DNA ligase (Gibco BRL, Eggenstein, Germany) was used to catalyze the formation of a phosphodiester bond between juxtaposed 5'phosphate and 3'hydroxyl termini in duplex DNA. Recircularization of vector DNA can be limited by adding a higher concentration of foreign DNA than of the plasmid vector in the ligation reaction. Therefore, vector and fragment, which were previously digested with the same or compatible restriction enzymes, were ligated in a molar ratio of 1:10 and incubated at room temperature for 30-60 minutes.

Reaction mix	:	<50 ng vector DNA
		<500 ng DNA fragment
		1 U T4 DNA ligase
		1x ligase buffer
		H ₂ O ad 20 µl

5x ligase buffer	:	(Gibco BRL, Eggenstein, Germany)
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3.1.4.3 Fill-in of restriction endonuclease ends

To ligate two DNA strands without compatible restriction sites, overhanging DNA ends were modified to form blunt ends using T4 DNA Polymerase (New England

Biolabs, Frankfurt am Main, Germany). This enzyme catalyzes the synthesis of DNA in the 5' → 3' direction to fill-in 5' overhangs and has a 3' → 5' exonuclease activity removing 3' overhangs to form blunt ends.

The fill-in reaction was incubated 15 min at 12°C, stopped by adding final 10 mM EDTA and heated for 20 min at 75°C.

Reaction mix:	1-2 µg DNA with protruding "sticky end"
	1x T4 DNA polymerase reaction buffer
	0.2 mM nucleotides
	1 U T4 DNA polymerase
	H ₂ O ad 100 µl

T4 DNA polymerase	
reaction buffer (10x):	50 mM NaCl
	10 mM Tris-HCl
	10 mM MgCl ₂
	1 mM dithiothreitol

3.1.5 Transformation of bacteria

Different methods of transformation were carried out to introduce plasmid DNA into bacteria. Therefore, appropriate competent cells have been prepared for transformation.

3.1.5.1 Preparation of electrocompetent cells from *S. typhimurium*

One liter of pre-warmed LB-broth (37°C) was inoculated with 10 ml of a fresh overnight culture. Bacteria were cultured at 180 rpm and 37°C until an optical density (OD₆₀₀) of 0.5-1 was reached. After cooling the bacterial culture (15-30 min on ice), the bacteria were harvested by centrifugation for 15 min at 4000x g and 4°C. The pellet was washed once with 1l ice-cold H₂O, afterwards with 500 ml ice-cold H₂O and finally with 20 ml ice-cold glycerin (10% v/v in H₂O). At the end, the bacterial pellet was resuspended in 2-3 ml ice-cold glycerin (10%) and aliquots (50 µl per lot) were stored at -70° until use.

3.1.5.2 Electroporation of *S. typhimurium*

S. typhimurium strains were transformed by electroporation of competent cells. Before electroporation, the plasmid DNA was dialyzed against water for 20 min. For transformation, 1 µg of DNA was added to 50 µl of competent bacteria and the suspension transferred into an ice-cold cuvette (2mm gap; BioRad, Munich, Germany). Electroporation parameters were 25 µF, 2.5 kV and 200 Ω (Gene Pulser II; BioRad, Munich, Germany). Subsequently, 1 ml SOC-medium was added to the cuvette and mixed. The suspension was transferred into an Eppendorf reaction tube, shaken for 1h at 37°C before plating on LB-agar plates containing the appropriate antibiotics. Plates were then incubated overnight at 37°C.

3.1.5.3 Preparation of PEG-competent cells of *E. coli* strains

5 ml LB-broth were inoculated with *E. coli* TOP 10 and incubated overnight at 37°C with agitation at 180 rpm. On the next day, 100 ml LB-broth were inoculated with 1 ml of the fresh overnight culture and grown at an OD₆₀₀ of 0.6-0.65. The bacteria were cooled for 10 min on ice, harvested by centrifugation at 1500x g for 10 min and at 4°C. The pellet was resuspended in 1 ml ice-cold solution A, 5 ml solution B was added and the suspension carefully mixed. Aliquots (100 µl per lot) were stored at -70°C.

Solution A : 10 mM MgSO₄
0.2% glucose
in LB-broth, sterilization by filtration

Solution B : 36% Glycerin
12% PEG 8000 (polyethylene glycol)
12 mM MgSO₄
in LB-broth, sterilization by filtration

3.1.5.4 PEG-transformation of *E. coli* strains

100 µl of the PEG-competent cells were thawed on ice, mixed with 10 µl of a ligation reaction or 1 µl of plasmid DNA and incubated for 30 min on ice. The bacteria were

then incubated for 1 min at 42°C and additionally 5 min on ice. 500 µl SOC-medium was added to the bacteria and the suspension was shaken for 30-45 min at 37°C. Finally, the bacterial suspension was plated on LB-agar plates containing selective antibiotics and incubated overnight at 37°C.

3.1.6 Bacterial cultures

Bacteria were grown on agar plates or in broth at 37°C. Liquid cultures of *E. coli* and *L. monocytogenes* were shaken at 180 rpm whereas recombinant *Salmonella* for oral genetic vaccination were cultured at 37°C and 50 rpm in LB-broth containing 1.5% NaCl (final) and supplemented with appropriate antibiotics when required.

3.1.7 Long-term storage of bacteria

To store bacteria for a long-term period, 700 µl of a fresh overnight culture was mixed with 300 µl glycerin (50% v/v in H₂O) and frozen at -70°C.

3.1.8 Polymerase chain reaction (PCR)

PCR (Mullis, 1986) offers the possibility to detect small amounts of specific DNA. The principle of PCR is based on the enzymatic amplification of a DNA segment between two regions of known sequence. Two oligonucleotides are used as primers, which bind to complementary DNA strands in opposite directions and flank the DNA segment that is to be amplified. Amplification occurs in a series of synthetic reactions that are catalyzed by a DNA polymerase. First, the double stranded template DNA is denatured by heating at 95°C. Then, the complementary oligonucleotide primers, which are present in excess, are annealed to the single stranded DNA under hybridization conditions (at ca. 50-70°C). Subsequently, the DNA dependent, heat-resistant DNA polymerase synthesizes complementary DNA sequences by extension of 3'-OH prime ends with nucleotides. Afterwards, the cycle of denaturation, annealing and extension is repeated several times. The specific DNA sequence can be amplified exponentially up to the factor 10^5 - 10^7 within 20 to 30 cycles

In this study, the AmpliTaq Gold™ (Perkin Elmer, Weiterstedt, Germany) or the Cloned Pfu DNA Polymerase (Stratagene, Heidelberg, Germany) were used as

polymerase for PCR. The AmpliTaq Gold™-Polymerase is a thermostable DNA polymerase derived from *Thermus aquaticus* that is supplied in an inactive form and was activated by incubation for 10 min at 95°C at the beginning of the PCR-program. This avoids unwanted by-products due to unspecific annealing of oligonucleotides and polymerase activity at lower temperatures.

The cloned Pfu DNA polymerase is isolated from the hyperthermophilic, marine archaebacterium *Pyrococcus furiosus*. The temperature optimum is at 72-78°C. Compared to the Taq-polymerase, the Pfu-polymerase possess 3' to 5' exonuclease proofreading activity that provides a 12x lower error rate in DNA synthesis than the Taq-polymerase.

The thermocycler PCRExpress (Hybaid, MWG, Ebersberg, Germany) was used for DNA synthesis.

Reaction mix : 1x PCR buffer
200 µM nucleotide mix
5-10 pmol primer 1
5-10 pmol primer 2
2,5 U AmpliTaq Gold™ oder Cloned Pfu DNA Polymerase
<1 µg DNA
H₂O ad 20 µl

Nucleotide mix (2 mM) : 100 mM stock solution of dATP, dCTP, dGTP, dTTP
(Pharmacia Biotech, USA) diluted in H₂O (1:50)

10x PCR buffer

for Taq-polymerase : 100 mM Tris-HCl (pH 8.3); 500 mM KCl; 15 mM MgCl₂;
0.01% (w/v) gelatin;

10x PCR buffer

for Pfu-polymerase : 200 mM Tris-HCl (pH 8,8); 100 mM KCl; 100 mM
(NH₄)₂SO₄; 20 mM MgSO₄; 1 % Triton X-100; 1 mg/ml
nuclease-free BSA (bovine serum albumin)

Reaction tubes : 0.2 ml (Applied Biosystems, Hamburg, Germany)

PCR conditions

Program	polymerase	denaturation	annealing	extension	Cycles
AmpR	Pfu	94°C; 20 sec	45°C; 30 sec	72°C; 2 min	32
pUCori	Pfu	94°C; 20 sec	45°C; 30 sec	72°C; 2 min	32
AmpR-pUCori	Pfu	94°C; 20 sec	45°C; 30 sec	72°C; 2 min	32
pMB1 replicon	Pfu	95°C; 1 min	45°C; 30 sec	72°C; 4 min	35
PTetR	Pfu	95°C; 1 min	45°C; 30 sec	72°C; 4 min	35
p15Aori	Pfu	95°C; 20 sec	45°C; 30 sec	72°C; 2 min	32
pSC101ori	Taq	95°C; 1 min	60°C; 1 min	72°C; 1 min	30
PCmR	Taq	95°C; 1 min	55°C; 1 min	72°C; 1 min	35

Purification of PCR products

Products from a PCR reaction were purified using the "QIAquick PCR-Purification Kit" (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

3.1.9 DNA Sequencing

The "ABI PRISM™ BigDye Terminator Cycle Sequencing Kit" (Perkin-Elmer, Weiterstedt, Germany), based on the chain-termination method of Sanger *et al.* (1977), was used for sequencing of double-stranded DNA. In this method, the DNA sequence of interest is amplified by a heat-stable DNA polymerase using a specific primer, but extension is terminated by the introduction of base-specific, fluorescent dye-labeled dideoxynucleoside triphosphates. PCR products were examined using polyacrylamide gels to discriminate between single-stranded DNA chains differing in length by a single nucleotide.

Reaction mix for PCR: 4 µl BIG Dye PREMIX; 1 µg DNA; 3.2 pmol primer; H₂O ad 20 µl

PCR-Programm: 96°C, 30 sec denaturation
 52°C, 15 sec annealing
 60°C, 4 min extension
 25 cycles

Sequence assessment and statistical analysis

Sequences were analyzed using SequencherTM 3.0 (Gene Codes Corporation, Michigan, USA).

3.2 Immunochemical methods

3.2.1 ELISA (enzyme-linked immunosorbent assay)

ELISA represents a highly sensitive and specific method for the quantitative detection of secreted proteins such as antibodies or cytokines. In the sandwich ELISA, the highly purified capture antibody is noncovalently adsorbed (“coated”) onto plastic microwell plates due to hydrophobic interactions. In turn, the immobilized antibodies capture soluble antigen from samples applied to the plate. After removal of unbound material, the captured antigen will be detected by a secondary antibody. This detection antibody is conjugated to an enzyme, which catalytically converts a chromogenic substrate. The level of colored product can be measured spectrophotometrically using an ELISA reader at an appropriate wave length.

96-well-microtiter plates (MaxisorbTM Immunoplaten, Nunc, Wiesbaden, Germany) were coated with 50 µl/well of 0.5 µg/ml purified protein in coating buffer overnight at 4°C to bind the primary antibody to the carrier. The next day, plates were washed 3x with wash buffer using an ELISA-washer (Dynatech, Denkendorf, Germany) and blocked by adding 200 µl of 5% FCS in wash buffer for 1 h at 37°C to saturate free binding sites. After additional washing, 50 µl of serum was added at 1:1000 dilutions in wash buffer with 2.5% FCS and incubated for another 3 h at 37°C. Binding was revealed by using peroxidase-conjugated anti-mouse IgG and OPD as substrate. Color reaction was stopped by addition of 1 M sulfuric acid and measured with an ELISA reader MR 500 (Dynatech, Denkendorf, Germany) at 490 nm.

Coating buffer : 0.1 M Na₂CO₃; 0.1 M NaHCO₃; pH 9.6 in H₂O

Wash buffer : 0.1% Tween 20 in PBS (according to Sambrook *et al.*, 1989)

OPD-substrate solution: 1 mg/ml O-phenyldiamine in substrate buffer, 1 μ l/ml H_2O_2

Substrate buffer : 0.2 M NaH_2PO_4 ; 0.1 M Na_3 -Citrat; pH 5.0

To evaluate the levels of specific antibodies against β -galactosidase or listeriolysin, standard ELISA was employed with serum from immunized mice.

detection of	1. antibody	Sample	2. antibody
mAb- β -Gal	0.5 μ g/ml β -Gal	Mouse serum	goat-anti-mouse IgG (h+l)
mAb-LLO	0.5 μ g/ml wt-LLO	Mouse serum	goat-anti-mouse IgG (h+l)

3.2.2 ELISPOT (enzyme-linked immunospot assay)

The solid-phase enzyme-linked immunospot assay presents a powerful technique for detecting and enumerating individual cells that secrete a particular protein *in vitro*. The ELISPOT assay is based on the sandwich ELISA and receives its specificity and sensitivity by applying high affinity capture and detection antibodies as well as enzyme amplification of the color reaction. The assay can be employed not only for analyzing specific antibody-secreting cells, but also for measuring the frequencies of cells producing and secreting other effector molecules like cytokines. In this study, the ELISPOT assay was utilized to determine and to evaluate antigen-specific, IFN γ -secreting CD8⁺ T cells from immunized mice.

Before the MAHA plates (Millipore Multiscreen-HA 96-well filtration sterile plates, Millipore, Bedford, MA) were coated with 50 μ l/well R4 monoclonal capture antibody (25 μ g/ml), they were moistened with PBS. After incubation overnight at 4°C with the capture antibody, the plates were washed five times with 200 μ l/well PBS and blocked by adding 100 μ l/well of IMDM medium containing 10 % FCS for 2 h at room temperature (RT). In the meantime, the restimulated lymphocytes were washed five times with medium; the number of viable cells was determined and adjusted to 2×10^6 cells/ml. From this cell suspension 50 μ l/well were added to the ELISPOT plate after discarding the blocking solution. Then, 50 μ l of peptide (2 μ g/ml) in IMDM compl. were added per well for stimulation. The β -galactosidase peptide aa877-885 or LLO peptide aa91-99 were used as specific antigen (depending on the vector taken for immunization) and the respective other peptide as negative control in each

3.3.3 Removal of adherent cells

Adherent growing cells have to be detached from the culture dish for counting and transfer to new culture flasks. Therefore, cells were washed once with PBS, removed enzymatically from the substrate by adding trypsin-EDTA solution (TE) and incubated at 37°C in a humidified atmosphere until the cells were detached. Immediately, TE was inactivated by addition of FCS containing medium. The cells were harvested by centrifugation, washed once with PBS and resuspended in medium

PBS : 137 mM NaCl; 2.6 mM KCl; 6.4 mM Na₂HPO₄ x 2 H₂O;
1.4 mM KH₂PO₄; in H₂O (pH 7)

TE : 0.5 g/ml trypsin; 0.2 g/ml EDTA; in PBS

3.3.4 Conservation of mammalian cells

Cells from a dense growing 12- or 6-well plate were taken in 0.5 ml ice-cold freezing medium. The cell suspension was temporarily frozen at -70°C and finally stored in liquid nitrogen.

Freezing medium : 10 % (v/v) dimethylsulfoxide (Merck, Darmstadt, Germany)
in FCS

3.3.5 Thawing of cells

The frozen cells were thawed at 37°C in a water bath and the cell suspension was taken up in medium. Afterwards, the cells were harvested by centrifugation, washed once and resuspended in medium

3.4 Calcium phosphate transfection

Transfection represents a method for the transfer of DNA into cell culture cells. The principle of calcium phosphate-precipitation is based on the formation of an insoluble precipitate of calcium phosphate, to which the DNA can be adsorbed. These calcium phosphate-DNA-complexes are taken up by the cells through endocytosis. The DNA

reaches the nucleus, where it can be transcribed (transient transfection). Some of the DNA can be integrated into the genome of the recipient cell (stable transfection), which can subsequently transmit this DNA segment to the next cell generation.

Practically: 3×10^5 adherent cells per 25 cm^2 culture flask were seeded the day before. The medium was exchanged for 5 ml of fresh medium 4 h before transfection. For preparation of the calcium phosphate/DNA coprecipitate, 250 μl of the CaCl_2 -DNA-mixture were added drop by drop under gentle mixing to 250 μl HEBS-buffer (2x) and incubated for 30-60 min at room temperature. The precipitate was then added to the medium above the cells and the transfected cells were cultured at 37°C . Excess of precipitate was removed the next day by exchange of medium.

HEBS-buffer (2x) : 280 mM NaCl; 50 mM HEPES; 1.5 mM $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$; ad 100 ml; adjust with 5 M NaOH to pH 7,1; sterile filtering, aliquots stored at -20°C

CaCl_2 -DNA-mixture : 25 μl CaCl_2 (250 mM); 10 μg cotransfer-DNA; H_2O ad 250 μl

3.5 Infection of cell lines with *S. typhimurium aroA*⁻

The day before infection with recombinant *S. typhimurium*, HEp-2 cells were seeded in 24-well plates at a density of 5×10^4 cells/well in IMDM medium without antibiotics. The recombinant *Salmonella* were grown overnight in LB broth containing 1.5% NaCl final at 37°C and 50 rpm up to an OD_{600} of ~ 0.8 . Then, the bacteria were washed once with PBS and resuspended in antibiotic-free IMDM medium. After 24 h the HEp-2 cells has reached a density of approximately 1×10^5 cells/well and were infected with an MOI (multiplicity of infection) of 200:1, 100:1 and 50:1 in a total volume of 200 μl . The MOI was confirmed by plating serial dilutions of the suspension on LB-agar plates. Cultures were centrifuged to enhance infection and incubated for 1-2 h at 37°C . Cells were washed three times with PBS and cultured in medium supplemented with 100 $\mu\text{g}/\text{ml}$ gentamicin (Biochrom AG, Berlin, Germany) for 1h to kill the remaining extracellular bacteria. Afterwards the medium was exchanged for IMDM compl. containing penicillin and streptomycin and supplemented with gentamicin to kill the intracellular and extracellular *Salmonella*, respectively.

3.5.1 Determination of intracellular bacterial numbers

In a parallel experiment, HEp-2 cells were infected as described above. One hour after incubation in gentamicin-containing medium, cells were washed three times with PBS and lysed for 30 min in 0.2% Triton-X 100 in H₂O. Colony forming units of lysates were enumerated by plating serial dilutions.

3.5.2 Detection of β -galactosidase by *in situ* staining of cells

Expression of β -galactosidase could be visualized in fixed cells after cleavage of the indicator substrate X-gal (5-brom-4-chlor-3-indolyl- β -D-galactopyranosid). HEp-2 cells infected with recombinant *Salmonella* (see 3.5) were stained for β -gal activity after 24 h and 48 h. The adherent cells were washed three times with PBS and air-dried for 30 min at room temperature. Afterwards cells were fixed with ice-cold acetone/methanol (1:1 v/v) for 5 minutes. The fixing solution was aspirated and the cells dried again before adding the freshly prepared X-gal substrate. Color reaction was developed by incubation overnight at 37°C in a humified atmosphere and β -gal-expressing cells were detected due to their blue color by light microscopy.

X-gal substrate:	K ₃ FeIII(CN) ₆	5 mM	(stock solution: 500 mM)
	K ₄ FeII(CN) ₆	5 mM	(stock solution: 500 mM)
	MgCl ₂	2 mM	(stock solution: 500 mM)
	X-gal	100 µg/ml	(stock solution: 10 mg/ml)

Stock solutions were stored at -20°C.

3.6 Immunization with recombinant *Salmonella*

3.6.1 *In vitro* plasmid stability

All *S. typhimurium* strains were cultured in LB-broth containing 1.5 % NaCl overnight at 37°C at 50 rpm until they reach OD₆₀₀ of 0.8 and passaged for 5 days in cultures with or without antibiotic selection. The percentage of stability was calculated by counting colony forming units (cfu) on antibiotic agar plates divided by total viable cfu on LB agar without selecting antibiotics, multiplied with 100.

3.6.2 Colonization and *in vivo* plasmid stability

At different time points after a single oral immunization with the recombinant *Salmonella* (see 3.6.3), Peyer's patches, mesenteric lymph nodes, spleen and liver were removed. Organs were homogenized in 1 ml 0.1% Triton X-100 at 10000 rpm using a homogenizer (Polytron PT 3000, Kinematica AG, Littau-Lucerne, Switzerland) to lyse the cells in the suspension. Serial dilutions were plated on LB-Agar with or without antibiotics and the cfu were determined after overnight incubation at 37°C to assess bacterial colonization and the percentage of plasmid stability.

3.6.3 Oral Immunization

For *in vivo* studies, *S. typhimurium aroA*⁻ were grown in LB-broth containing 1.5% NaCl (final) at 50 rpm and 37°C overnight until they have reached an OD₆₀₀ of 0.8. It was assumed that at an OD₆₀₀ of 0.8 1ml of culture contains approximately 5×10^8 bacteria. After the bacteria were washed 2x in PBS, they were resuspended in sodiumbicarbonate (5% w/v in PBS). The number of bacteria in solution was adjusted to $1-5 \times 10^8$ per 100 µl by measuring the optical density (OD₆₀₀) and was confirmed by plating serial dilutions of the suspension. Groups of mice were inoculated with $1-5 \times 10^8$ recombinant *Salmonella* intragastrically using a gavage needle (Acufirm #1428LL, Dreieich, Germany).

Mice received either a single or four doses of recombinant *Salmonella* in 21-30 day intervals and the induction of cellular immune responses were analyzed in spleen and lymphnode. To examine the induction of specific cytotoxic CD8⁺ T cells, the JAM-assay or ELISPOT for IFN γ production was performed. CD4 T cell proliferation was measured by a helper-T-cell assay using the WST method. To investigate humoral responses, the serum of mice was analyzed for antigen specific antibodies in ELISA.

3.6.4 Preparation and analysis of mouse serum

At different time points, mice were bleed from the tail vein or blood was taken from the heart after the mice had been sacrificed. To separate serum from the blood clot, the blood was centrifuged for 10 min at maximal speed in an Eppendorf centrifuge.

The serum was then stored at -20°C until it was investigated for antigen specific antibodies by ELISA.

3.6.5 Preparation and culture of T cells

At different time points after immunization, mice were sacrificed and spleen, Peyer's patches and mesenteric lymphnodes were isolated under aseptic conditions. Splenic T cells were isolated by washing out the spleen with 10 ml erythrocyte-lysis-buffer, centrifuged, washed once with IMDM complete and resuspended in the same medium. T cell were also prepared through gentle homogenization of mesenteric lymphnodes, washed and taken up in medium.

One part of the cells was used for proliferation assays and the JAM-assay *ex vivo*. The other part was cultivated in medium sized culture flasks with 15-20 ml medium for 5 days to use them after specific restimulation for ELISPOT.

Erythrocyte-lysis-buffer: 17 mM Tris; first dissolve in 800 ml H₂O (pH 7.6); add 140 mM NH₄Cl; and H₂O ad 1l; pH 7.2

3.6.6 Helper-T-cell assay

Isolated CD4⁺ T cells from immunized mice proliferate after addition of specific antigen *in vitro*. This cell proliferation and cell viability could be quantified by a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 (Roche, Penzberg, Germany). WST-1 is cleaved to formazan by mitochondrial dehydrogenases in viable cells. An expansion of viable cells results in an increase in the overall activity of these enzymes and subsequently leads to an increase in the amount of formazan dye formed. This is directly correlated to the number of metabolically active cells in the culture. The production of the formazan dye could be quantified by an ELISA reader by measuring the absorbance of the dye solution at an appropriate wavelength.

To carry out the cell proliferation assay after immunization, 6 x 10⁵ isolated lymphocytes from spleen and mesenteric lymphnodes per well were incubated in 96-

well-flat bottom tissue culture plates. The T cells were restimulated with 1-10 µg/ml of antigen in a final volume of 200 µl. After incubation of the plates for 24 h in a humidified atmosphere, 20 µl/well cell proliferation reagent WST-1 was added and incubated for another 4 h at 37°C and 5% CO₂. Then the plates were shaken thoroughly for 1 min on a shaker. The absorbance of the samples against a background control as blank was finally measured using an ELISA reader at 480 nm.

3.6.7 Cytotoxic T cell assay

To evaluate the induction of specific cytotoxic T cells after oral immunization, the JAM-assay was performed to directly measure cytotoxic activity of CD8⁺ T cells or the ELISPOT assay to determine the frequency of IFN γ -secreting CD8⁺ T cells in response to specific antigenic peptide.

Isolated lymphocytes from immunized animals were directly used in the JAM-assay. For ELISPOT assays, lymphocytes were restimulated *in vitro* for five days with 1 µg/ml specific antigen in IMDM compl. in culture flasks at 37°C and 5% CO₂. Therefore, cell cultures from individuals immunized with a β -galactosidase expressing construct were restimulated with the β -gal peptide and analogous listeriolysin primed cells with the CD8⁺-specific LLO peptide. After restimulation, the cells were harvested by centrifugation and analyzed by ELISPOT assay (see 3.2.2).

3.6.8 JAM-assay

The so-called JAM-assay (Matzinger, 1991) is a simple assay to measure DNA fragmentation and cell death to directly assess cytolytic activity. Proliferating target cells are labeled with [³H]thymidine by addition of [³H]thymidine into IMDM medium compl.. The radioactive thymidine is taken up by the cells and incorporated into the DNA during replication. Cytotoxic T cells can lyse these labeled target cells that release their DNA. Subsequently, free DNA is degraded into small fragments. After incubation of target cells with CTLs, cells were harvested by vacuum aspiration through fiberglass filters at a distinct time point. Only intact DNA from living cells is trapped in these filters, whereas small DNA fragments pass through. The more cells are killed, the less is the radioactivity on the filters. Thus, the radioactivity that is

measured corresponds to intact DNA and reflects the number of living targets that remain in each well.

Labeling of target cells

P815 cells in logarithmic growth phase were used as target cells. 3×10^5 cells were seeded in 1 ml IMDM medium compl. per well of a 12-well-plate. After addition of 5 μCi [^3H]thymidine per well, the cells were incubated for 4-6 h at 37°C in a humidified atmosphere to allow incorporation of radioactivity. Finally, the target cells were washed two times with medium to remove excess of radioactivity and resuspended in an appropriate volume.

Practical performance

This cytotoxic T cell assay was performed directly (*ex vivo*) with spleen cells in 96-well-plates with 10^4 labeled target cells in a total volume of 200 μl per well. Isolated spleen cells from immunized mice were used as effector cells in concentrations ranging from 10^5 to 10^6 cells/well to test different effector-to-target (E:T) ratios. After addition of 1 $\mu\text{g/ml}$ antigenic peptide to the cell mix, the target cells were incubated with effector cells at different ratios for 4 h. Cells were either immediately harvested or stored at -20°C until they were analyzed. For this, cells were transferred to fiberglass filters by vacuum aspiration using a cell harvester. The fiberglass filters were dried and shrink-wrapped after addition of liquid scintillation fluid to count them in a β -scintillation counter. As control, target cells alone or target and effector cells without specific antigen or with foreign antigen were used.

[^3H]thymidine TRA 310 : Amersham, Braunschweig, Germany

Cell harvester,

fiberglass filter : Inotech (Wallac, Turcu, Finland)

Scintillation fluid : Betaplate scint (Wallac, Turcu, Finland)

Scintillation counter : 1450 MicroBeta Trillux (Wallac, Turcu, Finland)

3.7 Infection of immunized mice with *L. monocytogenes*

Six to nine mice per group were challenged with 5×10^4 *L. monocytogenes* EGDe (10 x LD₅₀ for BALB/c mice) i.v. into the tail vein and observed during two weeks for survival. Practically, *Listeria* were incubated overnight at 180 rpm and 37°C in BHI broth. Next morning the culture was diluted 1:10 in fresh BHI broth and grown for approximately 2 h. Concentration of bacteria was determined by measuring the OD₆₀₀. Then the bacteria were washed in PBS and resuspended in PBS obtaining 5×10^5 *Listeria* per ml. Bacterial numbers were confirmed by plating serial dilutions of the suspension.

4 Results

At present, oral DNA immunization with attenuated *S. typhimurium aroA⁻* elicits a strong cellular immune response, consisting of CD4⁺ and CD8⁺ T cells, already after a single administration. In contrast, stimulation of humoral and protective responses was shown to require several applications of the vaccine (Darji et. al., 1997, 2000). The current vaccine consists of *S. typhimurium aroA⁻* carrying the commercially available pUC backbone-based pCMV β that drives antigen expression by the strong virally derived CMV promoter. The data obtained so far suggested that the amount of antigen expressed by this vector system was not sufficient *in vivo* to confer complete protection after a single dose of recombinant *Salmonella*. Therefore, the aim of this thesis was the improvement of the properties of the expression plasmid.

In the first approach, eukaryotic promoters should be selected that possibly allow higher antigen expression than the strong CMV promoter. The higher expression of antigen should improve cellular responses and especially the induction of antibodies, systemically and mucosally, as well as protective immunity.

From this first set of experiments, it became obvious that plasmid stability is the major problem in the improvement of *Salmonella*-based oral DNA vaccines. Generally, recombinant plasmids carry antibiotic resistance markers to facilitate identification and to allow plasmid retention *in vitro* by appropriate selection pressure. In the absence of stabilizing antibiotics, plasmids currently in use are unstable *in vitro* and *in vivo*. Since plasmid retention *in vivo* is critical for efficient gene transfer and, in addition, the application of antibiotic selection markers in the immunized host are not indicated, strategies needed to be developed to retain the expression plasmid in *Salmonella* without exogenous selection. Obviously, the high copy number of the expression plasmid pCMV β and its derivatives presently used in our laboratory put a heavy strain on the physiology of the *Salmonella* and the plasmid is lost rapidly without the stabilizing antibiotic. Lowering the copy number should consequently reduce the metabolic burden and hence improve plasmid retention.

The copy number of plasmid is usually regulated by the plasmids origin of replication (ori). Therefore, new low copy number expression plasmids were constructed

employing different ori's to stabilize the plasmid within *S. typhimurium aroA⁻* *in vitro* and especially *in vivo*.

4.1 Influence of different promoters on oral genetic immunization with *S. typhimurium aroA⁻*

Since it was demonstrated before that IFN γ down-regulates virally derived promoters, such as the strong CMV promoter (Qin et al., 1997), IFN γ that is produced during clearance of the *S. typhimurium aroA⁻* strain by the host possibly interferes with the activity of the CMV promoter after oral immunization. Therefore, a new series of recombinant *S. typhimurium aroA⁻* strains carrying plasmids that contain strong promoters of housekeeping genes or genes active in APCs were tested for their ability to induce specific immune responses. The expression of antigen driven by these promoters should be independent of the influence of IFN γ . In addition, the IRF-1 promoter was included in this series, since the IRF-1 promoter is activated by IFN γ . Recombinant *S. typhimurium aroA⁻* carrying an pUC-based eukaryotic expression plasmid encoding β -galactosidase as antigen under the control of the human elongation factor 1 α promoter (pEF1 β), the ubiquitin promoter (pUbi β), the macrophage-specific human lysozyme promoter (pLp β), the DC-specific CD11c promoter (pDCA β) and the human interferon regulatory factor-1 gene promoter (pIRF-1 β) were compared with the original human cytomegalovirus promoter (pCMV β) for the induction of specific CD4⁺ and CD8⁺ T cells. All of these new expression plasmids transformed in *S. typhimurium aroA⁻* were already available at the beginning of this study and kindly provided by A.I. Garbe.

From *in vitro* cultures, it became obvious that all of these expression plasmids were rapidly lost in the *Salmonella* carrier, although, the different plasmids were lost at various degrees. Whereas the pCMV β and pDCA β are maintained approximately up to 50% - 80% after an overnight culture in the presence of ampicillin, the other plasmids are nearly completely lost under these conditions (data not shown).

Nevertheless, groups of BALB/c mice were orally immunized by feeding 1×10^8 recombinant *Salmonella* with a pipette. 18 days later spleen cells were isolated and analyzed in the JAM-assay for cytolytic activity of antigen-specific CD8⁺ T cells (Figure 4.1). Apart from the plasmids containing the IRF-1 promoter and hLP

promoter, all constructs were similar to the original pCMV β plasmid and no one could induce stronger cytolytic activity of CD8⁺ T cells than the originally used plasmid (Figure 4.1). However, these results have to be interpreted with care, since the problem of plasmid stability (as discussed above) sheds some doubts on direct comparison of these constructs and might have influenced the induction of immune responses. Only if all plasmid construct would be stably maintained in the *Salmonella* carrier to the same extend, the influence of the different promoters on antigen expression and subsequently the induction of specific immune responses can be compared and evaluated.

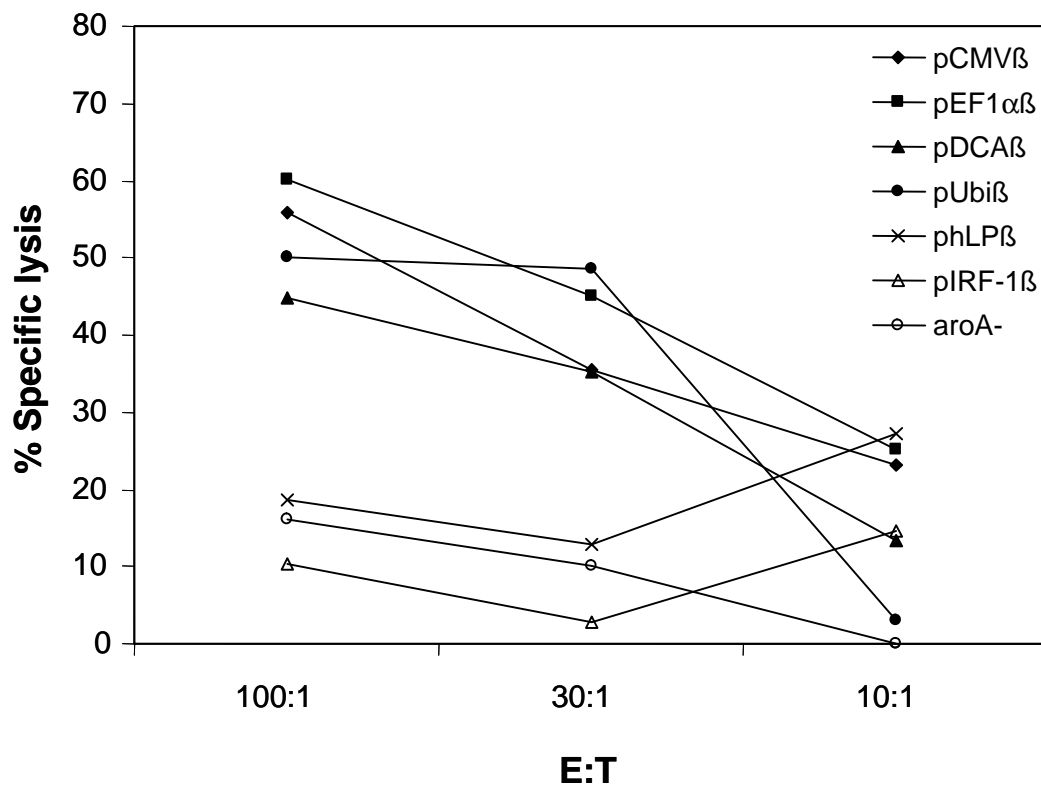


Fig. 4.1: Specific cytolytic activity of isolated splenocytes from BALB/c mice (ex vivo). Mice were orally immunized with a single dose of *S. typhimurium* aroA⁻ transformed with the original plasmid pCMV β or the new pUC-based expression plasmids pEF1 $\alpha\beta$, pDCA β , pUbi β , phLP β or pIRF-1 β containing different promoters. As negative control, mice received a single dose of *Salmonella* without plasmid (aroA⁻). Splenocytes were isolated 18 days post infection (p.i.) and tested with radiolabeled P815 cells for specific cytolytic activity. Different effector to target ratios (E:T) were analyzed after *in vitro* stimulation with β -gal peptide for 4 h in the JAM-assay.

4.2 *S. typhimurium* *aroA*⁻-mediated oral DNA vaccination with stabilized expression plasmids

To solve the stability problem encountered in the first set of experiments, a series of completely new expression plasmids was constructed that consist of exchangeable cassettes comprising origin of replication, selection marker and antigen coding region. After transformation of the *Salmonella* carrier with this series of plasmids, the influence of copy numbers on plasmid segregation *in vitro* and *in vivo* was examined. In addition, the ability of these transformants to induce cellular and humoral immune responses as well as their ability to confer protection against a challenge with a bacterial pathogen was compared with the original eukaryotic expression plasmid. Moreover, the influence of different antibiotic selection markers on the induction of immune responses were analyzed due to their different contents of immuno-stimulatory CpG motifs.

4.2.1 Construction of novel expression vectors

First, the high copy number plasmid pCMV β carrying the lacZ gene under the control of the human CMV promotor was modified in a way that the resistance cassette and the origin of replication (ori) could be exchanged at will by the introduction of new unique cloning sites. Therefore, the whole part containing the ampicillin resistance gene and the ori was removed from pCMV β by digestion with EcoRI and Sall. Then the ampicillin resistance gene and the pUCori were newly derived by PCR with primers containing several unique restriction sites 5'- and 3'-prime of the gene. In a second PCR, both sequences were fused together and the fragment was completed with the expression part of pCMV β including the CMV promotor, the gene for β -galactosidase and the polyA site after ligation via EcoRI and Sall resulting in the high copy number plasmid pCMV β m1A (Fig. 4.2A, B). Subsequently, the low copy number replicon pMB1 and p15A as well as the very low copy number ori of pSC101 were amplified including the same restriction sites as used for the pUCori of pCMV β m1A. The pUCori was then exchanged with these fragments giving pCMV β m2A, pCMV β m3A and pCMV β m4A, respectively (Fig. 4.3). Additionally, the tetracycline resistance gene was amplified by PCR and used to replace the ampicillin resistance gene in the particular plasmids. Thus, further resistance marker for

experimental analysis was generated and its influence on the stability of the expression plasmid could be investigated. The resulting plasmids were named e.g. pCMV β m2T, when the tetracycline resistance was introduced instead of ampicillin. Moreover, for protection studies, the *hly* gene coding for listeriolysin of *L. monocytogenes* was incorporated instead of *lacZ* as antigen into pCMV β m2A resulting in pCMVhlym2A. Figure 4.3 displays schematically the whole series of expression plasmids that were constructed, including the expected copy numbers.

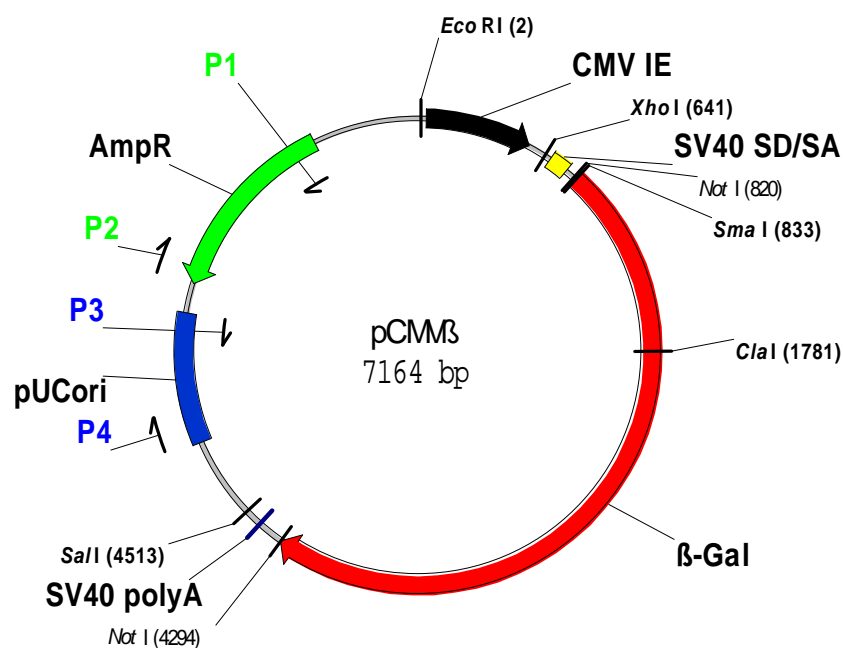
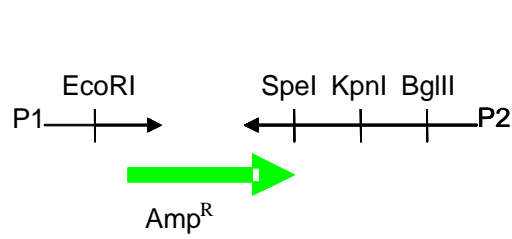


Fig. 4.2A: Construction of pCMV β m1A: Primer-pairs for the amplification of AmpR (P1/P2) and the pUCori (P3/P4) are indicated in the plasmid-card of pCMV β by arrows. For later cloning of pCMV β m1A, the pCMV β -backbone containing the CMV promoter and the *lacZ* gene for β -galactosidase expression was isolated by digestion with EcoRI and SalI.

First step:a) amplification of Amp^R 

b) amplification of pUCori

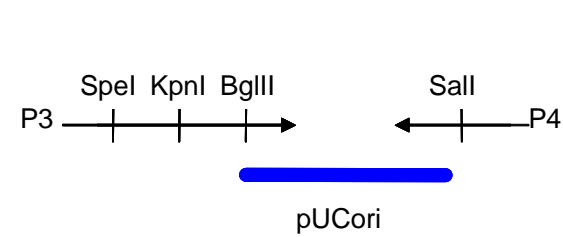
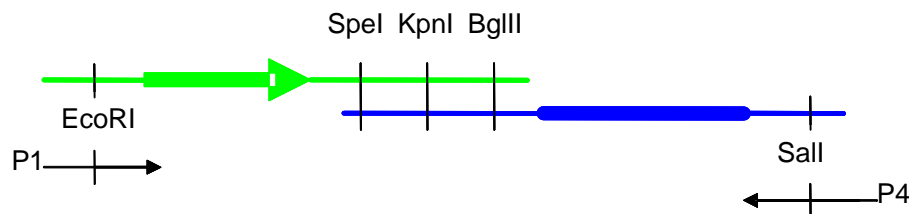
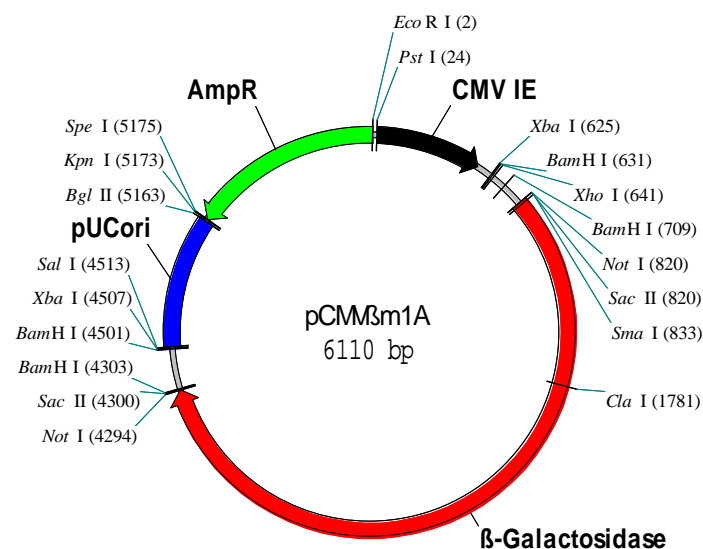
**Second step:**Fusion of Amp^R and pUCori by overlaying extension PCR.**Third step:**Ligation of the fused Amp^R -pUCori-fragment with the pCMV β -fragment containing the CMV promoter and the gene for β -galactosidase expression via EcoRI and SalI.

Fig. 4.2B: Construction of pCMV β m1A. The ampicillin resistance gene (Amp^R) and the pUCori were amplified by PCR to introduce several restriction sites fused by overlaying extension PCR and were completed with the lacZ expression cassette (β -galactosidase) from pCMV β resulting in pCMV β m1A.

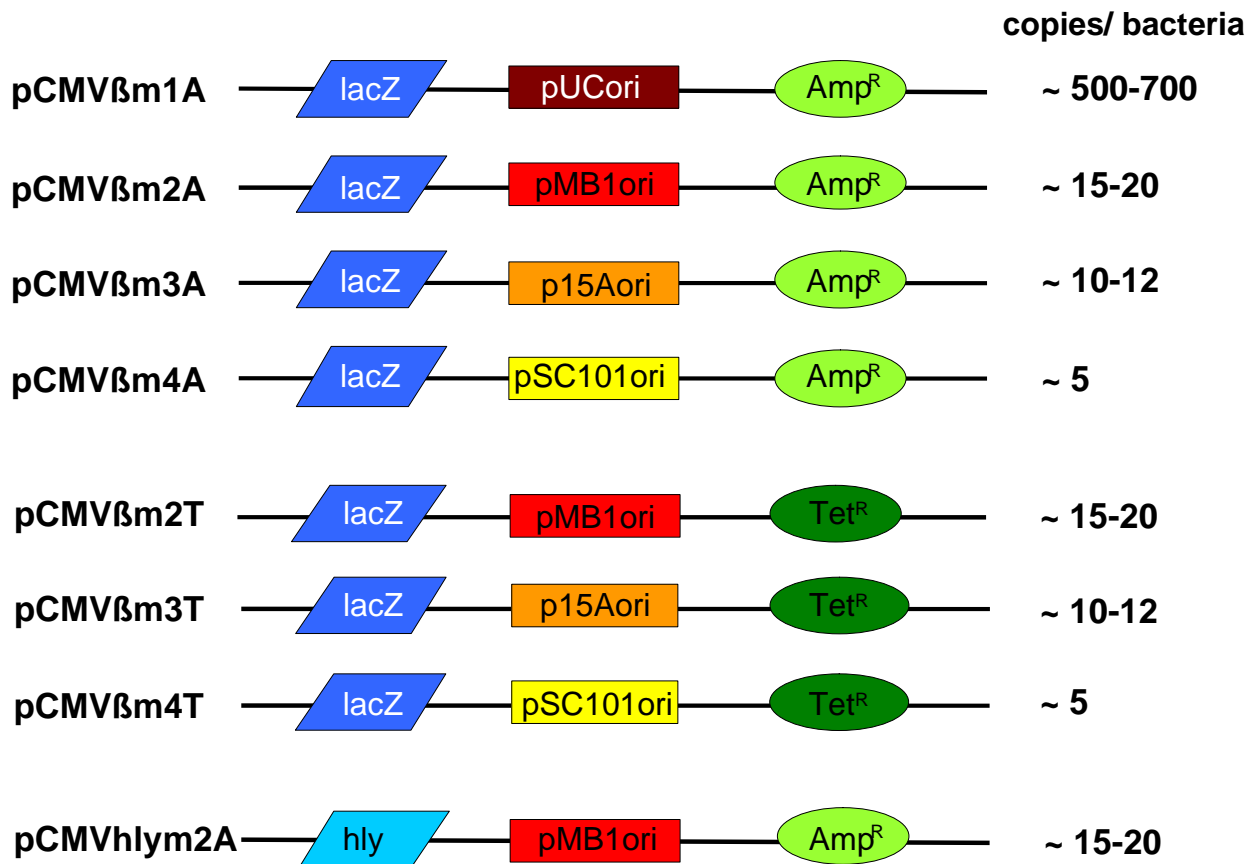


Fig. 4.3: Overview of plasmid constructs generated in this study.

4.2.2 Transformation of *S. typhimurium aroA*⁻

All expression vectors were introduced in *S. typhimurium aroA*⁻ by electro-transformation. Successful transformation was proven by isolation of plasmid DNA from mini-plasmid preparation and analytical digestion of the isolated plasmid. Since the *Salmonella* have a high nuclease activity, the additional wash step 7 was performed according to the manufacturer's protocol. All tested clones contained the appropriate vector (data not shown). One clone from each transformation was chosen to set up a stock culture and stored at -70°C.

4.2.3 Determination of plasmid copy number

To examine the number of plasmids, which are present in one bacterium, strains of *E. coli* and *S. typhimurium aroA⁻* carrying either the high copy number plasmid pCMV β , the low copy number plasmids pCMV β m2A and pCMV β m3A or the very low copy number vector pCMV β m4A were grown overnight at 37°C until OD₆₀₀ of ~0.8 was reached. Plasmid DNA was extracted from 3 ml of the overnight culture by mini-plasmid preparation. The cfu/ml were determined by plating on LB-agar plates containing antibiotics. To calculate the copy number, the amount of DNA solution was standardized to 5×10^8 bacteria (in the case of pCMV β 5×10^7 bacteria). The plasmids were digested with NotI and ClaI to obtain two fragments (2513 bp and 961 bp) of the β -galactosidase gene, which are identical in each construct. Then, the concentration of DNA was estimated by optical comparison with marker DNA after agarose-gel-electrophoresis (Fig. 4.4) and the copy number (see table 4.1) was calculated as follows:

1. Estimation of DNA concentration by optical comparison with marker DNA using the formula:

$$1 \text{ pmol of } 1000 \text{ basepairs (bp) DNA} = 0.66 \text{ } \mu\text{g}$$

2. Calculation of the number of molecules present in a single bacterium using the formula:

$$1 \text{ } \mu\text{g of } 1000 \text{ bp DNA} = 1.52 \text{ pmol} = 9.1 \times 10^{11} \text{ molecules}$$

$$\frac{\text{DNA concentration [pmol]} \times 9.1 \times 10^{11} \text{ molecules}}{1.52 \text{ pmol}} = \text{number of molecules/sample}$$

$$\frac{\text{number of molecules/sample}}{\text{number of bacteria/sample}} = \text{copies/bacterium}$$

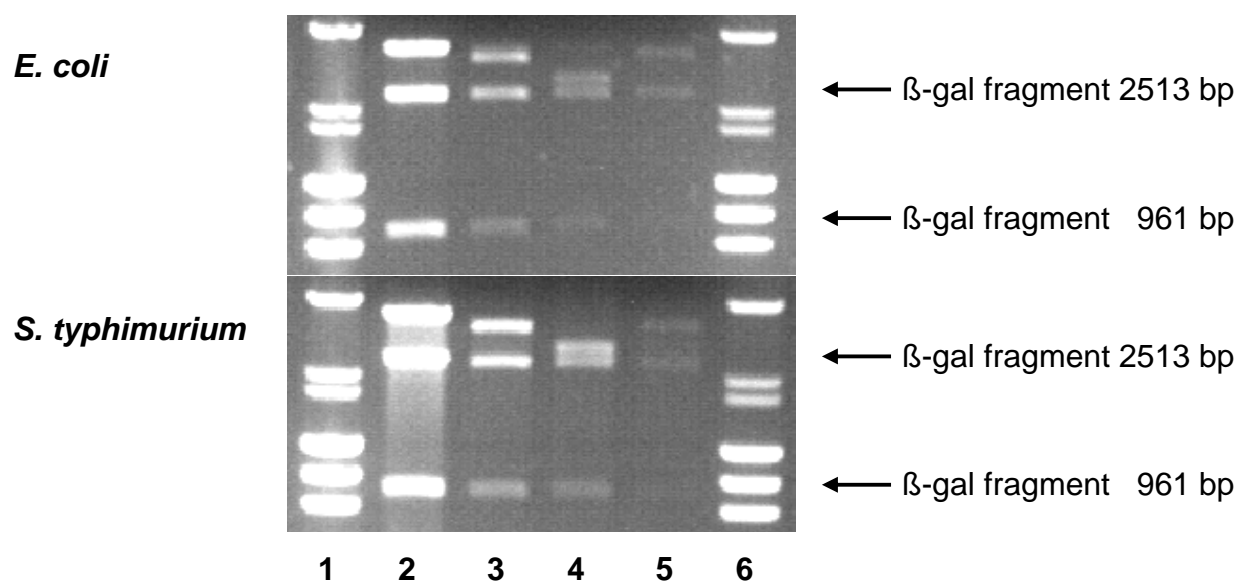


Fig. 4.4: Determination of plasmid copy number. Lane 1 and 6: marker DNA; lane 2: pCMV β ; lane 3: pCMV β m2A; lane 4: pCMV β m3A; lane 5: pCMV β m4A.

Table 4.1: Calculation of plasmid copy number.

Plasmid	Host strain	copy number/bacteria
pCMV β	<i>E. coli</i> DH5 α	~ 650
pCMV β m2A	<i>E. coli</i> TOP 10	~ 13
pCMV β m3A	<i>E. coli</i> TOP 10	~ 8
pCMV β m4A	<i>E. coli</i> TOP 10	~ 4
pCMV β	<i>S. typhimurium</i> aroA ⁻	~ 1120
pCMV β m2A	<i>S. typhimurium</i> aroA ⁻	~ 24
pCMV β m3A	<i>S. typhimurium</i> aroA ⁻	~ 14
pCMV β m4A	<i>S. typhimurium</i> aroA ⁻	~ 4

4.2.4 *S. typhimurium*-mediated transfection of an established cell line

Since gene-transfer *in vitro* with the original pCMV β plasmid was only shown efficiently in primary macrophages (Darji et al., 1997), but not in cell lines, it was investigated whether the increased plasmid stability in *Salmonella* could enhance the efficiency of gene-transfer. Hence, to compare the various new recombinant *Salmonella* strains in their ability to invade mammalian host cells and to mediate gene-transfer *in vitro*, the established HEp-2 cell line was infected.

Infection of HEp-2 cells with the various recombinant *Salmonella* described above was carried out with an MOI (multiplicity of infection) of 200:1, 100:1 and 50:1 in a total volume of 200 μ l. The MOI was confirmed by plating serial dilutions of the bacterial suspension on LB-agar plates. Cultures were centrifuged to enhance infection and incubated for 2 h at 37°C. Afterwards, extracellular bacteria were killed by addition of gentamicin to the cell culture. *Salmonella*-mediated gene transfer was assessed by X-gal staining of β -galactosidase expressing cells 24 h post infection. Successful transfer of DNA was detected by cytoplasmatic staining of HEp-2 cells by light microscopy (Fig 4.5). Since the CMV promoter in *Salmonella* shows residual expression of β -galactosidase, gene transfer has taken place only in cells that exhibit a homogenous X-gal staining of the cytoplasm, as shown in figure 4.5E.

Surprisingly infection of HEp-2 cells with *Salmonella* carrying pCMV β resulted in high numbers of transfectants, while recombinant *Salmonella* harboring low copy number plasmids show only few β -gal expressing cells. This was unexpected from results obtained in our lab (Darji et al., 1997) and by others (Grillot-Courvalin et al., 1999). However, only freshly thawed HEp-2 cells showed this phenomenon that was lost after several passages in culture. For reasons of time, it was not possible to further investigate this phenomenon.

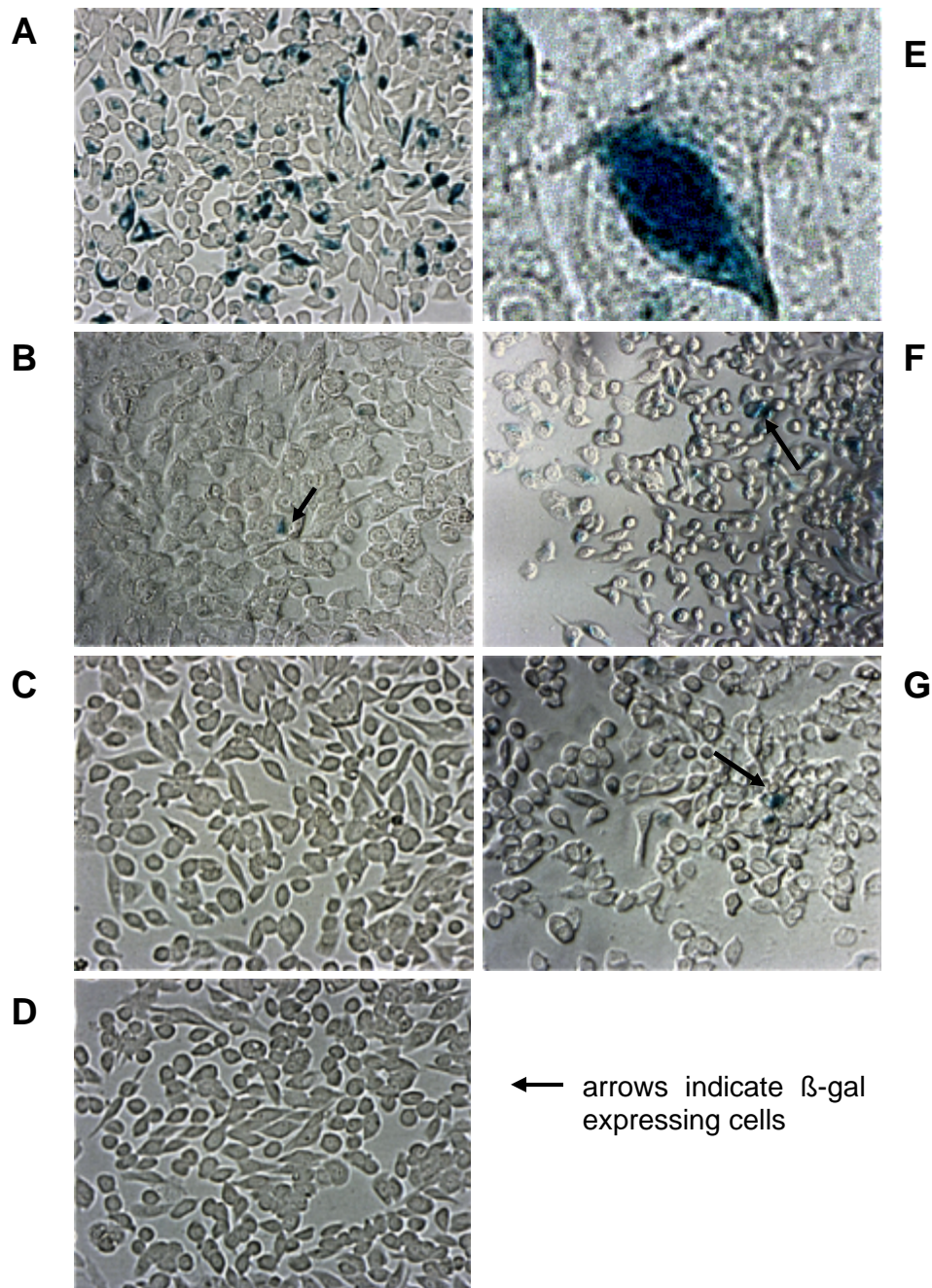


Fig. 4.5: Expression of β -galactosidase in HEp-2 cells after transfection by *S. typhimurium aroA*⁻ (*S. t.*). HEp-2 cells were infected at an MOI of 200:1 for 2 h with *S. t.* carrying either the expression plasmid pCMV β (A, E), pCMV β m2A (B), pCMV β m3A (C), pCMV β m4A (D), pCMV β m2T (F) or pCMV β m3T (G). 24 h p.i. cells were fixed and incubated with the indicator substrate X-gal. Expression of β -galactosidase activity was screened by light microscopy. Homogeneous staining of the whole cell is shown at a higher magnification after transfer of pCMV β from *S. t.* (E).

4.3 Plasmid stability

4.3.1 Growth curve

First of all, it was important to know how the new expression plasmids influence the growth behavior of *Salmonella typhimurium aroA⁻* *in vitro*. The different copy number of plasmids present in the bacterium as well as plasmid size should represent a varying burden to *Salmonella* and might differentially interfere with their metabolism and viability. To test this, LB-broth containing the appropriate antibiotic was inoculated with a small amount of the transformants from the cryo-stock and incubated at 37°C and 180 rpm. At different time points, the optical density (OD₆₀₀) was measured and the cfu/ml were determined by plating serial dilutions (Fig. 4.6).

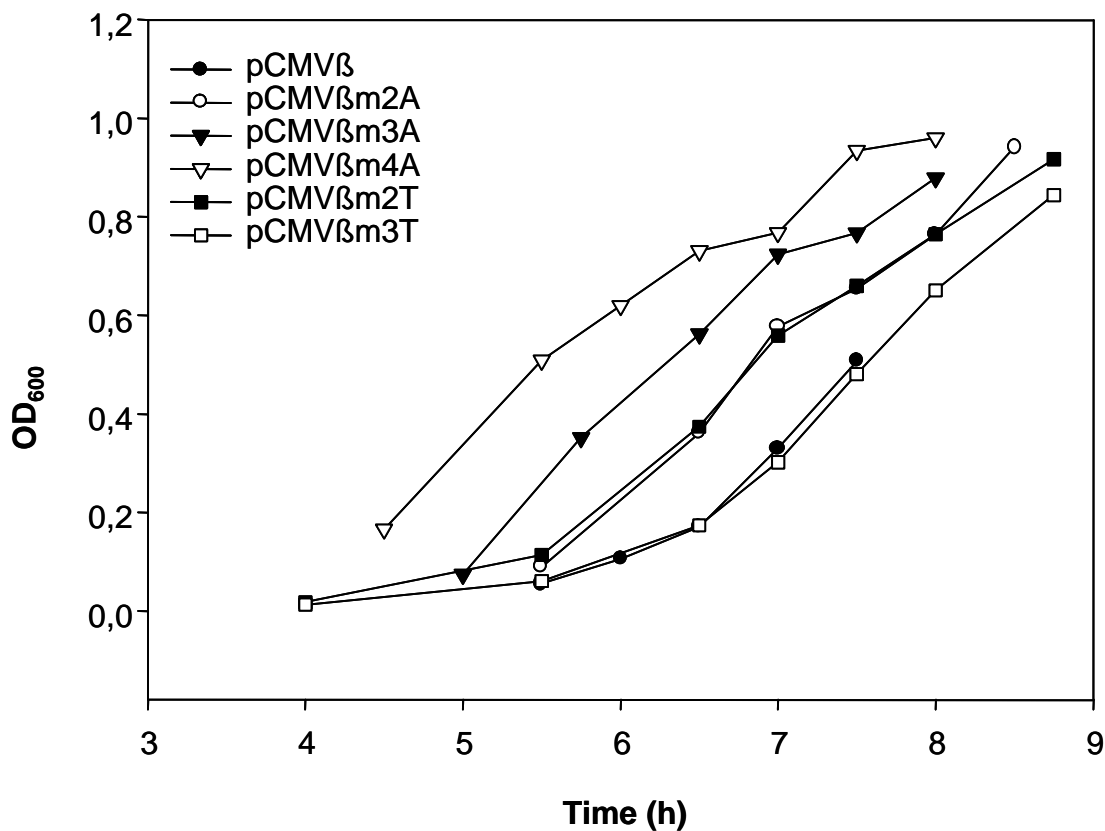


Fig. 4.6: Growth curve of *S. typhimurium aroA⁻* transformed with different eukaryotic expression plasmids encoding β -galactosidase.

As shown in figure 4.6 all plasmid-bearing *Salmonella* have a similar growth rate in the presence of the corresponding antibiotic regardless whether they carry a high or low copy number vector. Furthermore, the number of viable bacteria during growth was examined by plating serial dilution on LB-agar containing appropriate antibiotics (Fig. 4.7). Thus, under aerobic conditions the various plasmids obviously do not interfere with viability and growth.

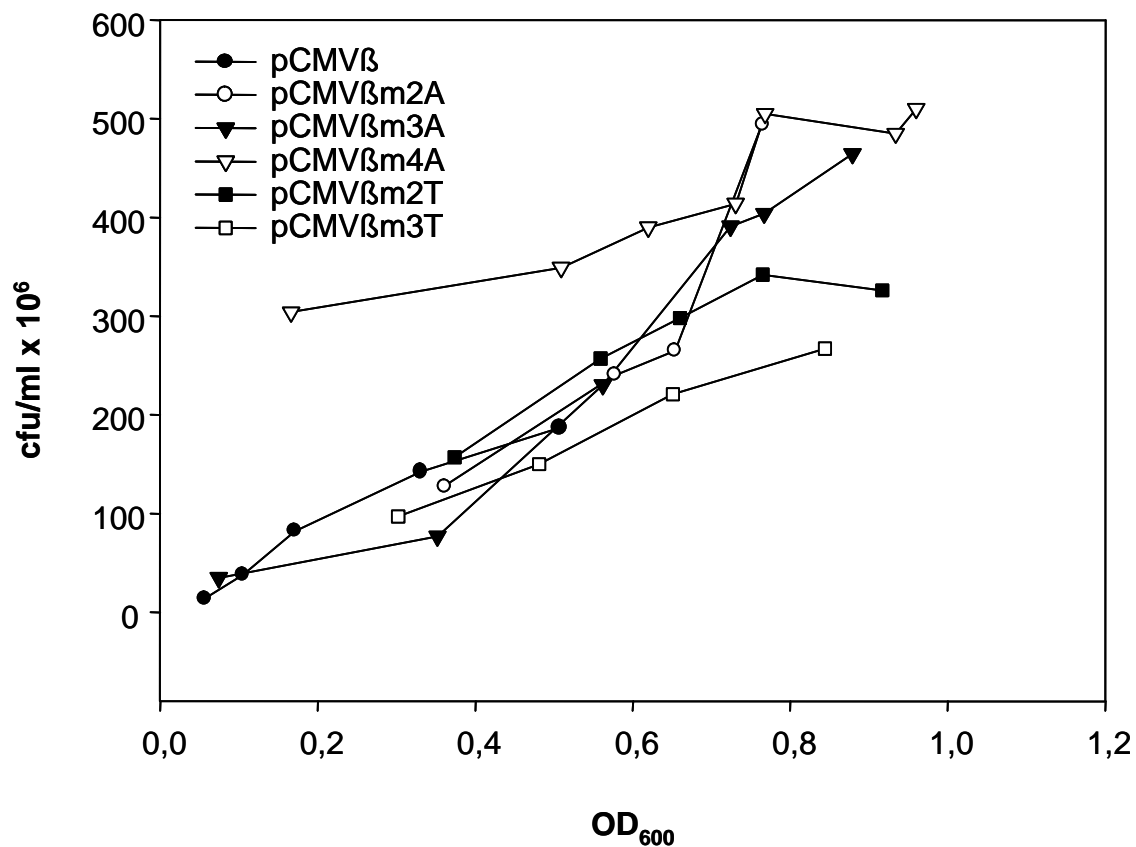


Fig. 4.7: Determination of colony forming units of recombinant *Salmonella* *in vitro*. At different time points during growth of *Salmonella* harboring the original high copy number plasmid pCMVβ or the newly derived low copy number eukaryotic expression plasmids, aliquots of the bacterial suspension were plated in serial dilutions on LB-agar plates containing the appropriate antibiotics. The cfu/ml were enumerated and plotted against the corresponding optical density (OD).

4.3.2 Plasmid stability *in vitro*

From the growth curve in the presence of antibiotics and determination of cfu in the bacterial culture no obvious differences were observed. However, it was more important for this study to investigate the plasmid stability *in vitro* of *S. typhimurium* *aroA*⁻ transformants under anaerobic conditions as used for vaccination experiments. Therefore, the recombinant *Salmonella typhimurium* *aroA*⁻ were grown in LB-broth with or without antibiotic selection for several cycles of overnight cultures to analyze

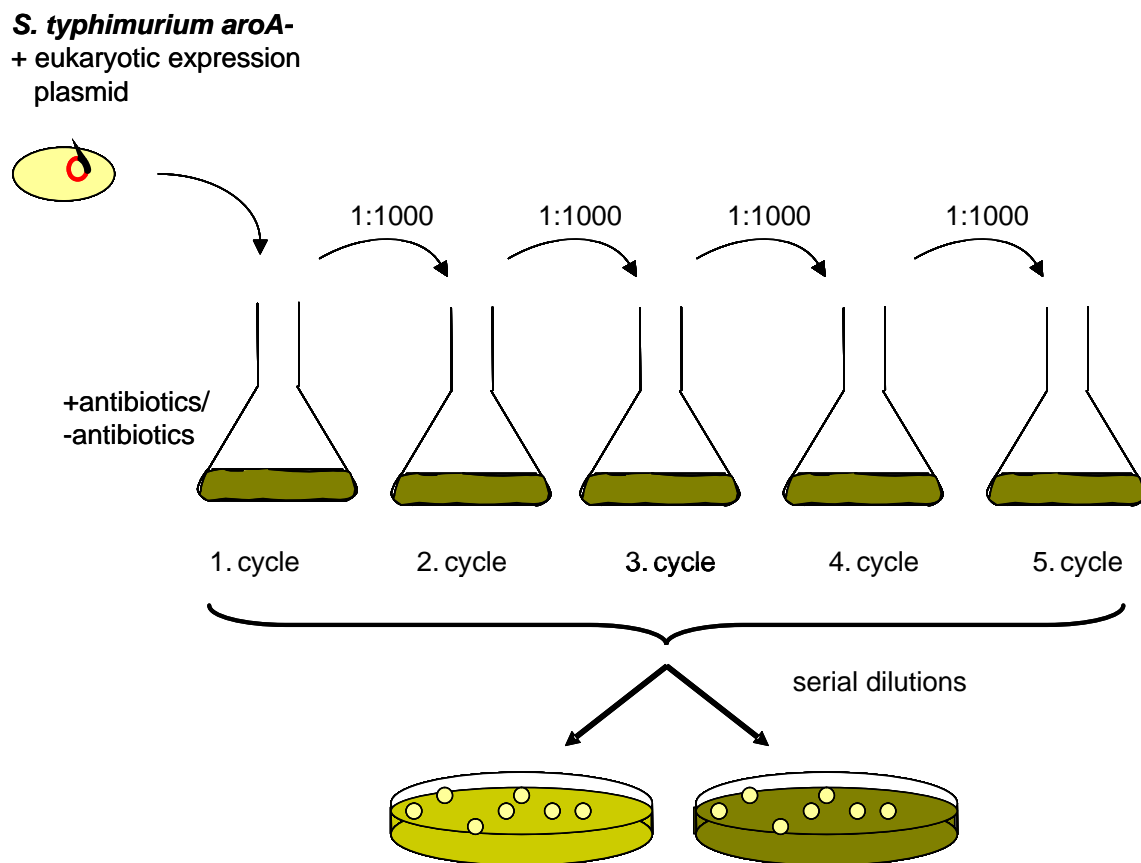


Fig. 4.8: Experimental schedule to test plasmid stability of *Salmonella* transformants *in vitro*. In each cycle, bacteria were grown in LB-broth with and without antibiotics under anaerobic conditions at 50 rpm overnight up to an OD₆₀₀ of ~0.8. For the next cycle, bacterial suspensions were diluted 1:1000 in fresh broth. From each cycle, serial dilutions were plated on LB-agar with and without selective antibiotics and bacterial colonies were counted to calculate plasmid stability.

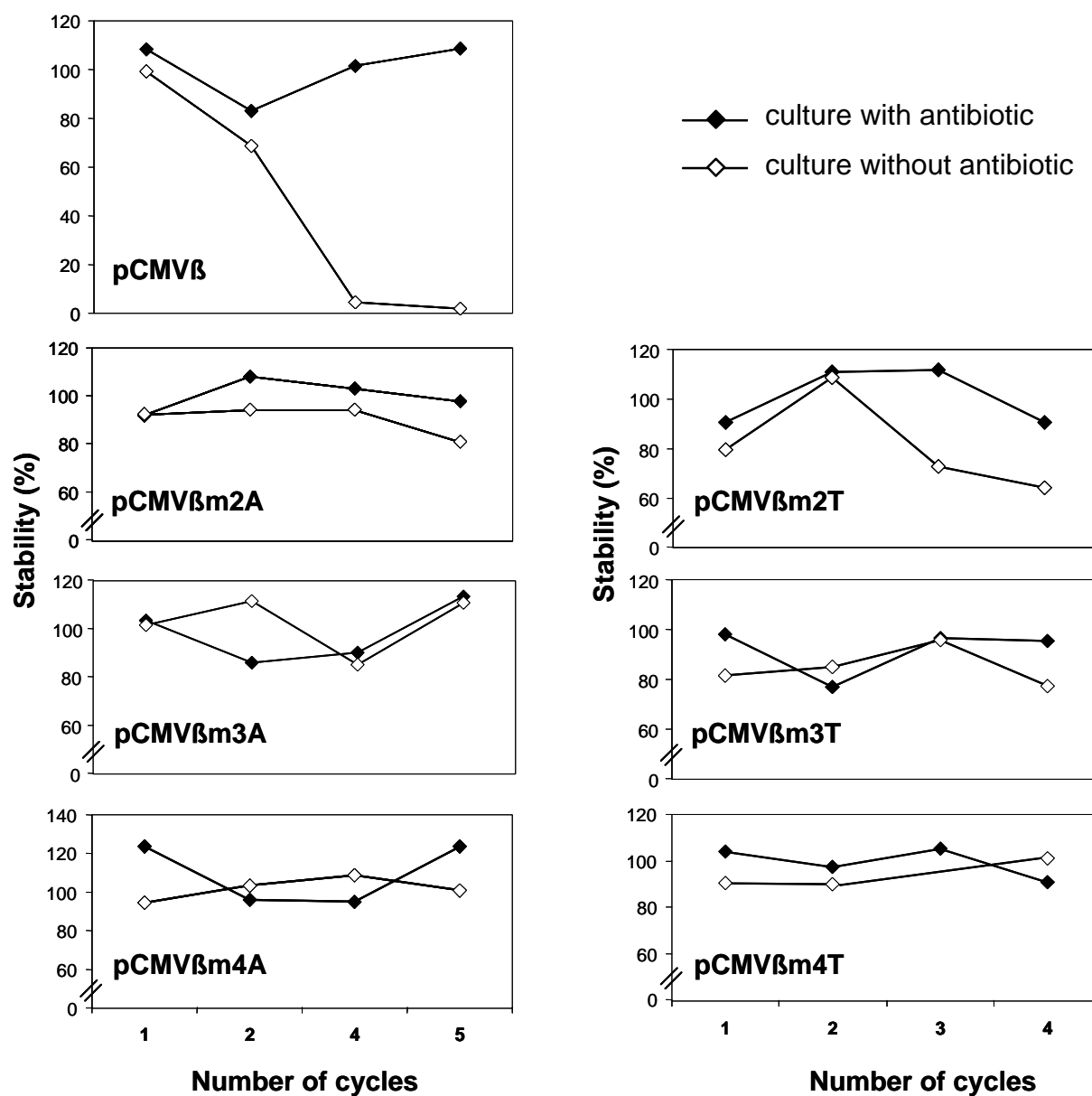


Fig. 4.9: Plasmid stability *in vitro*. Recombinant *Salmonella* were grown in culture with and without the appropriate antibiotic. Plasmid stability was calculated by plating on LB-agar with and without antibiotics.

the effect of an antibiotic-free environment on plasmid segregation at each cycle. Serial dilutions were plated on LB-Agar or LB-Agar containing selective antibiotics. Colony forming units were enumerated and the percentage of plasmid stability was calculated. The procedure of this experiment is schematically depicted in Figure 4.8. Antibiotic resistance was taken as indicator for the presence of the expression plasmid.

As shown in Fig. 4.9, the high copy number plasmid pCMV β is rapidly lost, when the selective antibiotic is omitted, whereas addition of ampicillin resulted in nearly 100% plasmid stability over time. In contrast, the low copy plasmids pCMV β m2A and pCMV β m3A as well as pCMV β m4A (Fig. 4.9) are stably maintained in the *Salmonella* even without any selective antibiotic. Furthermore, low copy plasmids carrying the tetracycline resistance gene instead of ampicillin were also stably retained in *Salmonella* (Fig. 4.9). This is a very important step for the use of such vaccines, as they can be easily handled for large-scale production in fermenters.

4.3.3 Plasmid stability *in vivo*

Most important for the improvement of these novel eukaryotic expression plasmids for *Salmonella*-mediated gene transfer was their stabilization *in vivo*, since this most likely represents the strongest influences on immune-inductivity.

To test this, groups of mice received a single oral dose of $1-5 \times 10^8$ recombinant bacteria intragastrically (i.g.) and bacterial colonization of murine tissues and plasmid retention *in vivo* was investigated over 21 days. At different time points, the spleen, mesenteric lymph nodes, Peyer's patches and liver were removed from immunized mice and bacterial colonization and plasmid stability was assessed by plating the homogenized tissues on LB-agar plates with and without antibiotics (Fig. 4.10).

The enumeration of bacterial colonies revealed that all *Salmonella* strains, including *S. typhimurium aroA*⁻ carrying pCMV β , were capable of colonizing Peyer's patches, mesenteric lymphnodes, spleen and liver. *Salmonella* expanded exponentially until day 7 to 10 and their numbers decreased up to day 21. Mesenteric lymphnodes and spleen were colonized with similar kinetics as Peyer's patches but with 10- to 100-fold lower number, respectively. Colony forming units in liver were similar to spleen (Fig. 4.11).

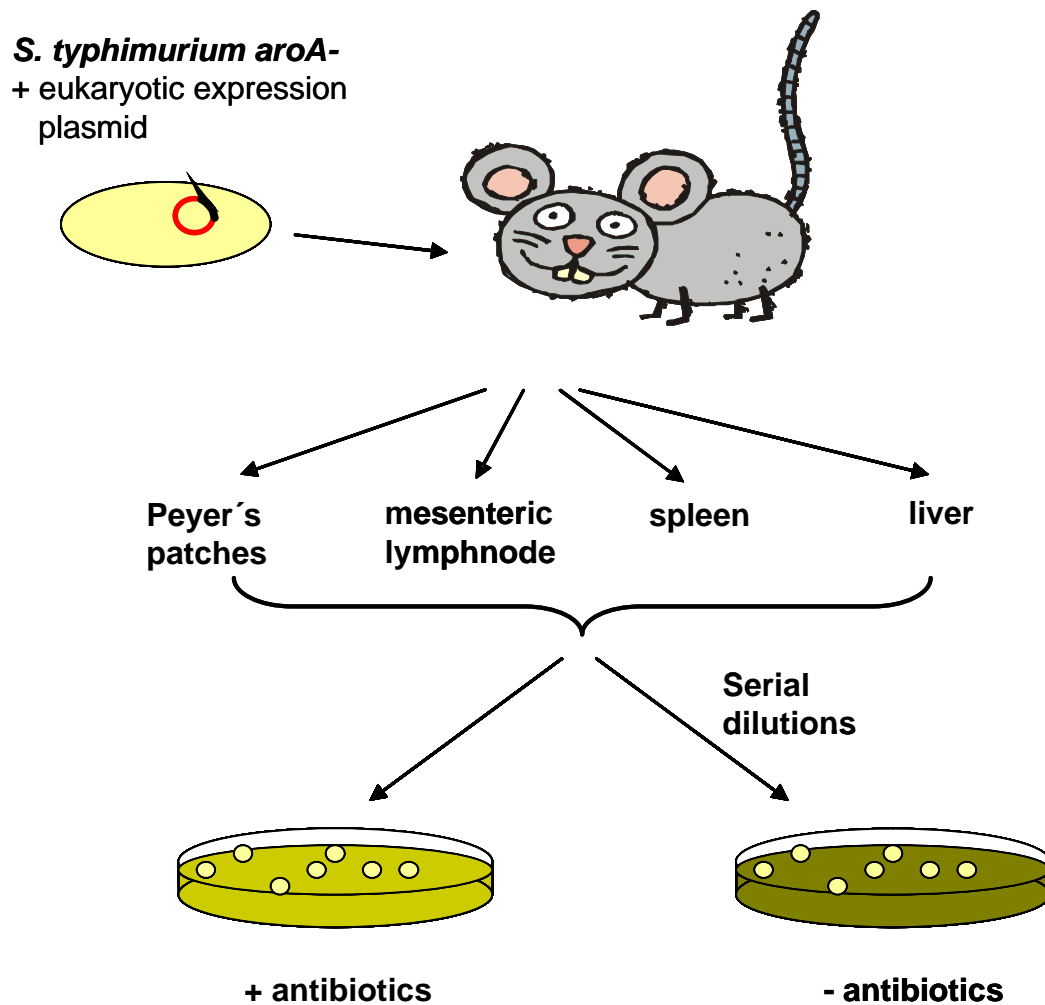


Fig. 4.10: Illustration of the working schedule to test colonization of murine tissues and plasmid stability *in vivo*. At day 2, 7, 10, 14 and 21 after inoculation of $1-5 \times 10^8$ recombinant *Salmonella* i.g., organs were removed and the cfu/organ were determined after plating serial dilutions.

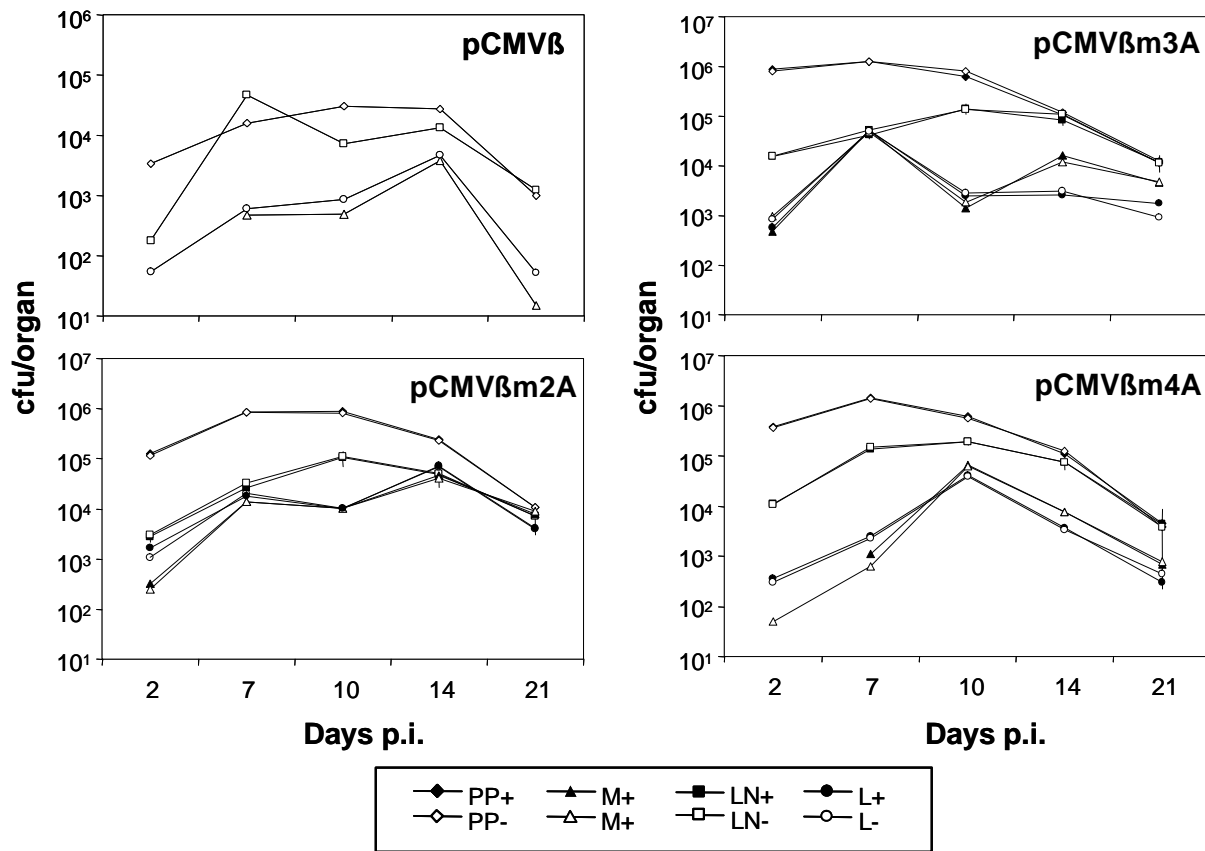


Fig. 4.11: Colonization of plasmid-bearing *S. typhimurium aroA⁻* in murine tissues. Peyer's patches (PP), mesenteric lymphnodes (LN), spleen (M) and liver (L) were removed on day 2, 7, 10, 14 and 21. Single cell suspensions were lysed and plated in serial dilutions on LB-agar with (closed symbols) or without (open symbols) 100 µg/ml ampicillin to determine the cfu/organ.

In contrast to the new strains with the low copy number plasmids, *S. typhimurium* carrying the high copy number plasmid pCMVβ were detected at a three fold lower number in all tissues, although the mice had received the same number of bacteria (Tab. 4.2). This observation confirms that the high copy number expression vector pCMVβ represents a high metabolic burden for *Salmonella*, consequently reducing their ability to colonize murine tissues.

Tab. 4.2: Bacterial recovery in Peyer's patches after oral application of recombinant *S. typhimurium* strains.

expression plasmid	Cfu/mouse	recovery ¹⁾
pCMV β	4×10^8	3.02×10^6
pCMV β m2A	4×10^8	8.62×10^6
pCMV β m3A	4×10^8	7.82×10^6
pCMV β m4A	4×10^8	14.13×10^6

¹⁾ only data from day 7 (day 10 for pCMV β) p.i. are shown

Subsequently, plasmid stability *in vivo* of the newly constructed expression vectors was calculated from these data and compared with the multicopy pCMV β . As demonstrated in figure 4.12, the lower number of copies stabilizes the plasmids carried within the *Salmonella*. As already mentioned, *S. typhimurium aroA*⁻ carrying the low copy number vectors could be detected in the Peyer's patches, mesenteric lymphnode and spleen as well as in the liver (Fig. 4.11). The plasmids pCMV β m2A, pCMV β m3A as well as pCMV β m4A were retained absolutely stable throughout the whole observation period of 21 days. In contrast, the pCMV β was obviously lost within the first two days, since no *Salmonella* carrying pCMV β could be detected at the first time point that was analyzed. Taken together, these results show that lowering the copy number in the eukaryotic expression plasmids resulted in an increase of plasmid stability *in vitro* as well as *in vivo*.

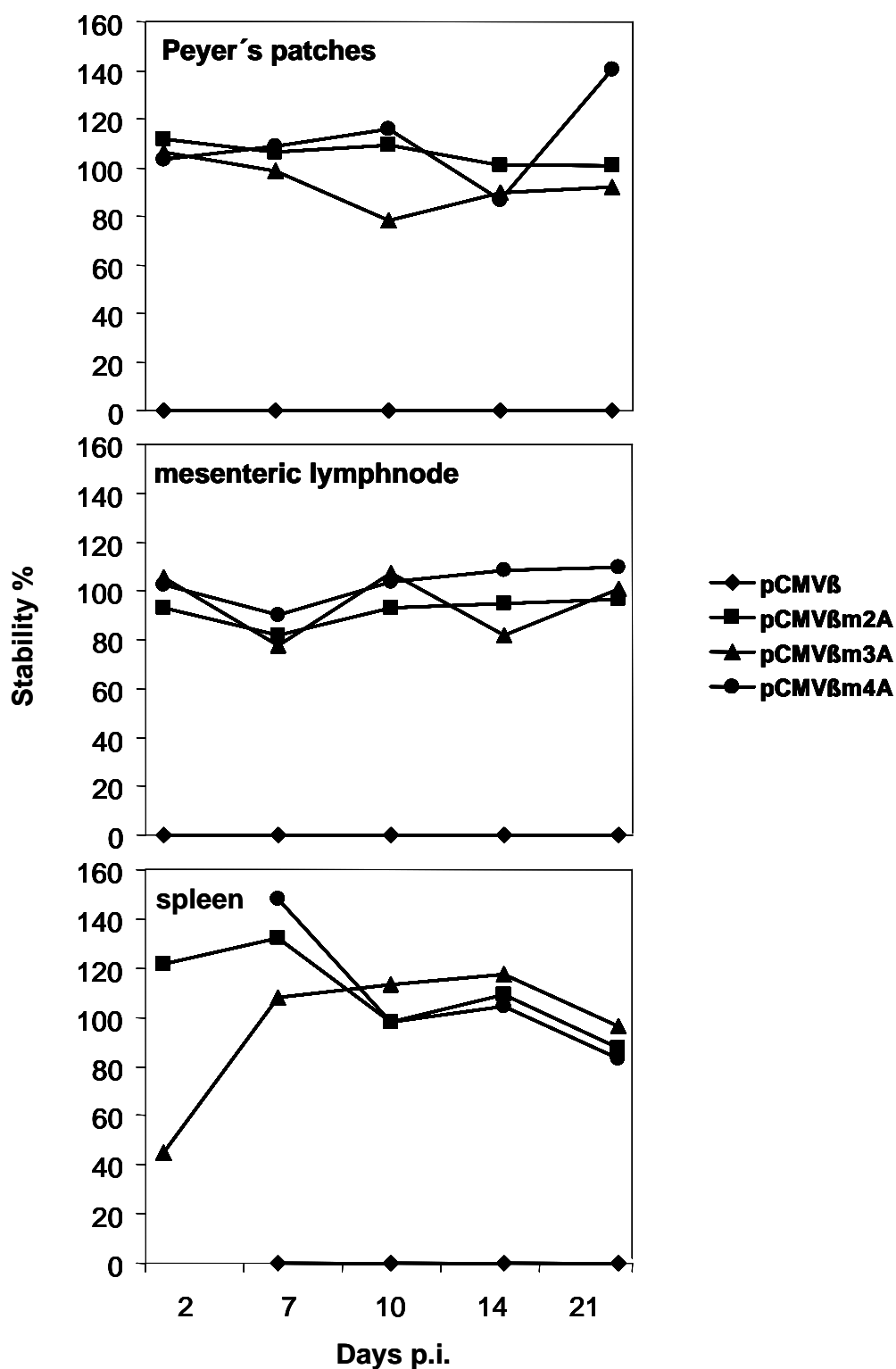


Fig. 4.12: Plasmid stability *in vivo*. Some of the values at day 2 diverge from 100% stability due to the extremely low numbers of cfu at this time points. These values most likely represent experimental errors rather than significant numbers of bacteria that do not carry the expression plasmid.

4.4 Immune responses elicited by oral immunization

The lower plasmid burden, due to the lower copy numbers, solved the problem of stability of the recombinant *S. typhimurium aroA⁻*. However, it was questionable whether this results in sufficient antigen expression to trigger an immune response in all three specific arms of the immune system, namely CD8⁺ and CD4⁺ T cells as well as antibodies. Therefore, groups of BALB/c mice were vaccinated intragastrically with either a single or four doses of 1×10^8 *S. typhimurium aroA⁻* carrying the original pCMV β or one of the newly established expression plasmids in a total volume of 100 μ l sodiumbicarbonate buffer per mouse using a gavage needle. Control mice were treated the same way with *S. typhimurium aroA⁻* without any expression plasmid. At various time points after immunization, mice were sacrificed, blood was collected and lymphocytes were isolated from spleen and mesenteric lymphnodes for analysis of specific immune responses. The induction of cytotoxic T cells, helper T cells and antibodies were analyzed by IFN γ -specific ELISPOT, proliferation assay and antigen specific ELISA, respectively. The general procedure of oral immunization for *Salmonella*-mediated gene transfer in mice and analysis of immune responses was illustrated in figure 4.13.

4.4.1 CTL response

To assess the response of CD8⁺ CTLs, lymphocytes from spleen and mesenteric lymphnodes of BALB/c mice were analyzed (Fig. 4.14). The isolated lymphocytes were restimulated *in vitro* for 5 days in IMDM medium containing 1 μ g/ml β -gal peptide aa 877-885 at 37°C in a humidified atmosphere. Cells were harvested by centrifugation, washed 5 times with PBS, resuspended in IMDM medium and the number of viable cells was determined. 1×10^5 cells per well of a 96-well-ELISPOT plate were then stimulated with the specific β -gal peptide or the LLO peptide aa 91-99 as a negative control for another 18 h at 37°C. Afterwards, the cells were lysed and IFN γ production was detected by binding of peroxidase-conjugated IFN γ specific antibodies. The development of the color reaction was stopped after 30-60 min and single spots were counted visually by a microscope.

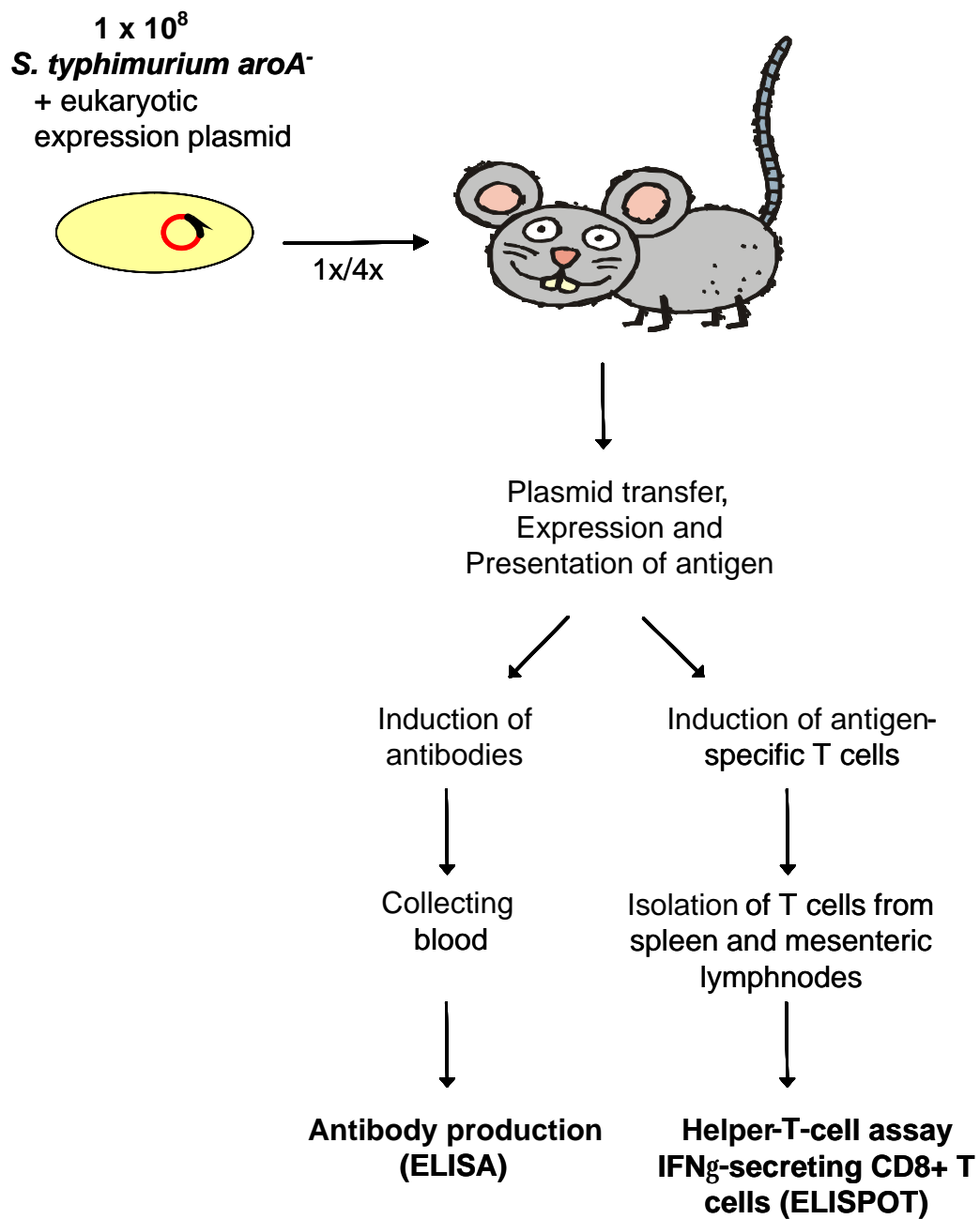


Fig. 4.13: Scheme of *Salmonella*-mediated oral DNA vaccination and analysis of immune responses that are elicited.

All *Salmonella* strains carrying low copy number plasmids, independent of the antibiotic resistance marker, provoked already after a single immunization a strong and specific cytotoxic T cell activity. This response was stronger for all low copy plasmid-carrying strains compared to the strain carrying the high copy number plasmid pCMV β (Fig. 4.14A). Four immunizations enhanced the number of IFN γ producing cells after administration of *Salmonella* carrying pCMV β significantly, while the response raised by *Salmonella* transformants with the different low copy number plasmids increased only slightly (Fig. 4.14B). The same results were obtained using low copy number constructs, where the tetracycline resistance gene was inserted instead of the ampicillin resistance gene (Fig. 4.14).

4.4.2 Helper T cell response

Spleen cells as well as lymphocytes from mesenteric lymphnodes of immunized mice, already tested for cytotoxic activity in ELISPOT, were additionally tested in a helper T cell assay for specific proliferation of CD4⁺ cells. As antigen, β -galactosidase protein was used for the restimulation of the specific response, whereas LLO protein served as negative control (data not shown). The proteins were taken at concentrations of 0.3, 1 and 3 μ g/ml and 3×10^6 cells/well were stimulated in 200 μ l medium, containing the protein antigen for 24 h. Specific proliferation was investigated by incorporation of WST for 4 h and color reaction was measured at OD₄₈₀.

Similar to the IFN γ response of CTLs, after only a single dose proliferation of CD4⁺ T cells from spleen as well as from lymphnode was much stronger after administration of *Salmonella* carrying the low copy number vectors compared to the high copy plasmid pCMV β (Fig. 4.15A). Multiple applications could just boost the helper T cell response in mice which received pCMV β -bearing *Salmonella*, but not or only weakly in the case of the low copy plasmids. Obviously, using the low copy number plasmid transformants, the maximum of the specific response is already obtained using a single administration.

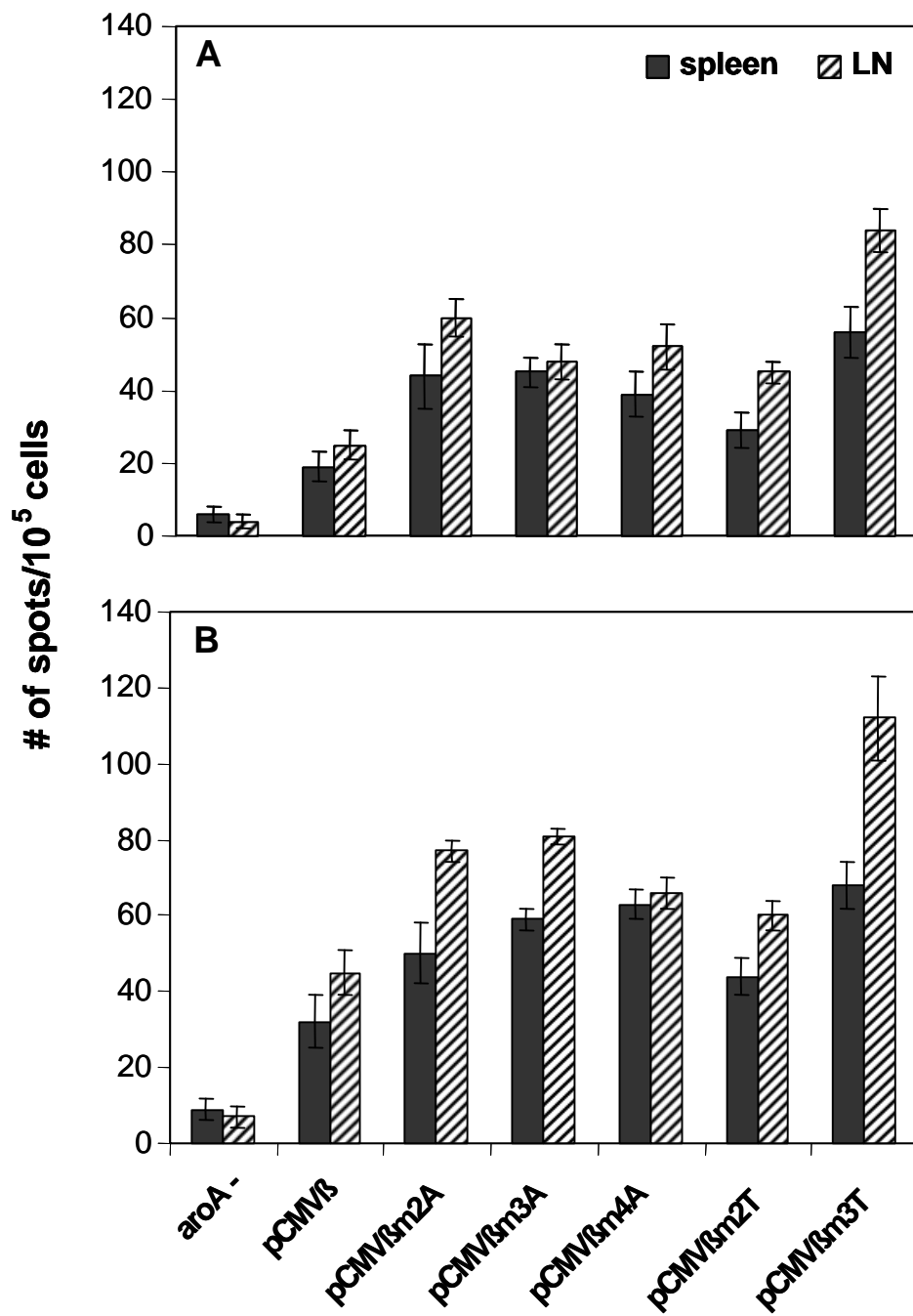


Fig. 4.14: ELISPOT on IFN γ -secreting CD8 $^+$ T cells. Groups of mice that received either a single (A) or four doses (B) of recombinant *Salmonella* were analyzed for the induction of antigen-specific CD8 $^+$ T cells by IFN γ -secretion *in vitro*.

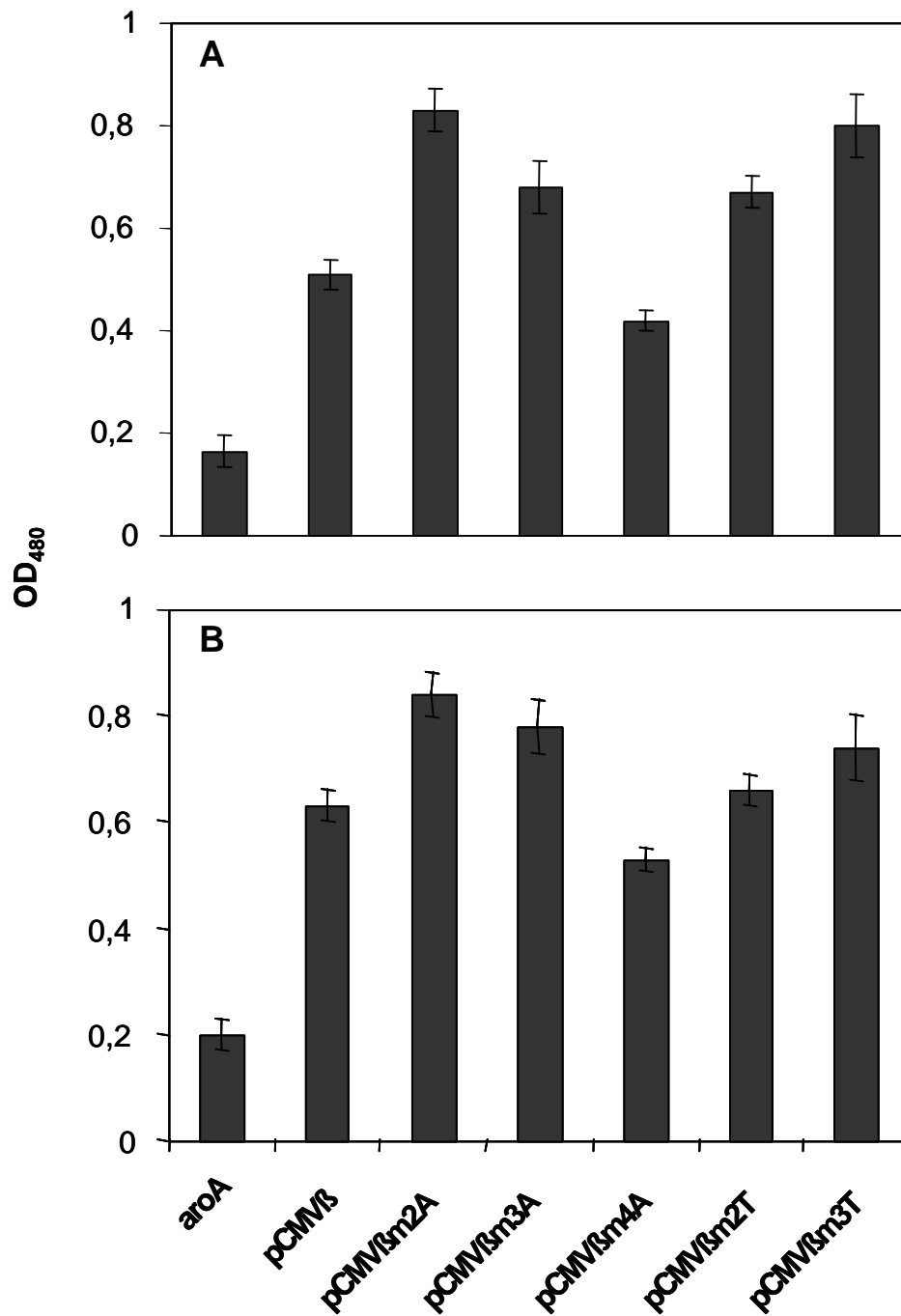


Fig. 4.15: Proliferation of antigen-specific CD4⁺ T cells in spleen. After single (A) or four applications (B), CD4⁺ T cells were analyzed for antigen-specific proliferation following *in vitro* stimulation with 3 µg/ml β-gal peptide.

4.5 Antibody response

The antibody response so far was the most problematic with the original system based on pCMV β . Therefore, it was interesting to see how strong the antibody response, raised by the new strains, appeared. The analysis of specific antibody responses by ELISA were performed on serum samples taken simultaneously from immunized mice sacrificed for the cellular assays either 144 days after a single or 37 days after the fourth administration. Serum from mice immunized with *S. typhimurium* without plasmid was taken as negative control. High β -galactosidase-specific antibody titers could be detected in serum of mice inoculated with only a single dose of recombinant *Salmonella* carrying the low copy number vectors, whereas pCMV β -bearing *Salmonella* evoke only a moderate antibody response (Fig. 4.16A). Several applications of any of the recombinant *Salmonella* strains could not significantly boost the specific antibody titer against β -galactosidase (Fig. 4.16B).

Since pooled sera from mice that received recombinant *Salmonella* carrying either the high copy number plasmid pCMV β or the very low copy number vector pCMV β m4A showed variable antibody titers against β -galactosidase, we analyzed sera from individual mice. Serum was collected from mice after a single administration of 1×10^8 recombinant *Salmonella* four and eight weeks post infection (p.i.). In this experiment, ELISA from both time points was carried out at different days and the titers from both experiments might not be absolutely comparable. However, it became clear that pCMV β -bearing *Salmonella* provoke low antibody titers in all individuals, whereas *Salmonella* transformed with pCMV β m4A elicit high antibody titers only in some of the mice at the early time point. But at the later time point, all of the mice appeared to respond by antibody production (Fig. 4.17).

Taken together, comparative studies between the high copy number plasmid pCMV β and the different low copy number plasmids, carrying the pMB1 replicon, p15A or pSC101 ori on the pCMV β backbone, revealed a dramatic increase in plasmid stability *in vitro* and *in vivo*. Furthermore, the increased stability resulted in a stronger induction of antigen-specific CD4 and CD8 T cells as well as antibody responses already after a single application. Moreover, antibody responses that are induced by

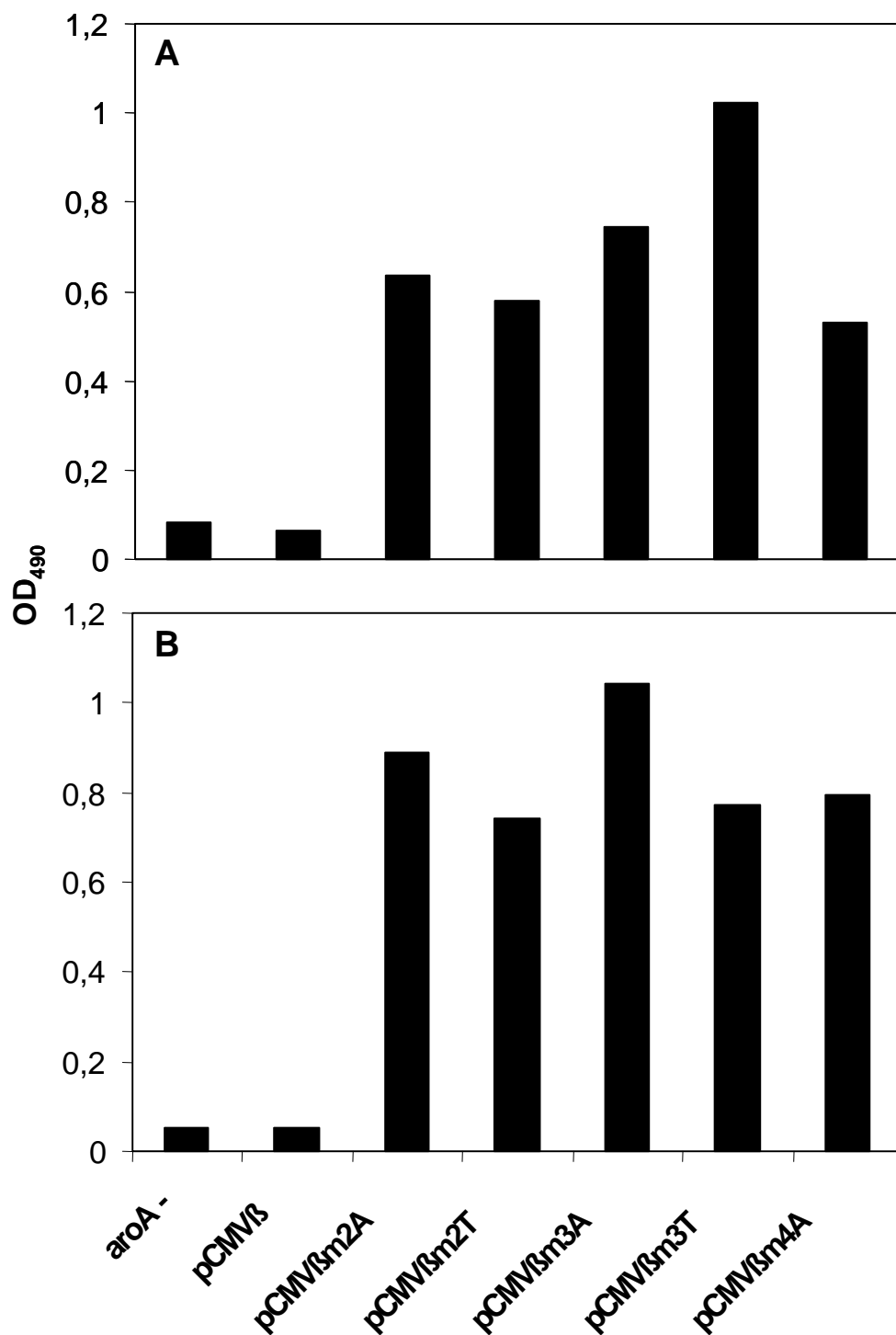


Fig. 4.16: Analysis of the antibody response. Serum samples of mice orally immunized either once (A) or four times (B) with recombinant *Salmonella* were examined for antigen-specific antibody titers against β -galactosidase in ELISA. Serum samples were taken 144 days after a single and 37 days after the fourth administration.

the new recombinant *Salmonella* strains were remarkably long-lived, since high titers of β -galactosidase-specific antibody titers could be detected still five month after a single oral dose. Such a strong and long-lived antibody response was never observed by the original system (Darji et al., 1997, 2000). This suggests that expression of low amounts of antigen after gene-transfer from *Salmonella* harboring low copy number vectors during a long period of time could prime stronger immune responses than do high amounts of antigen from multicopy plasmids present for a short time only.

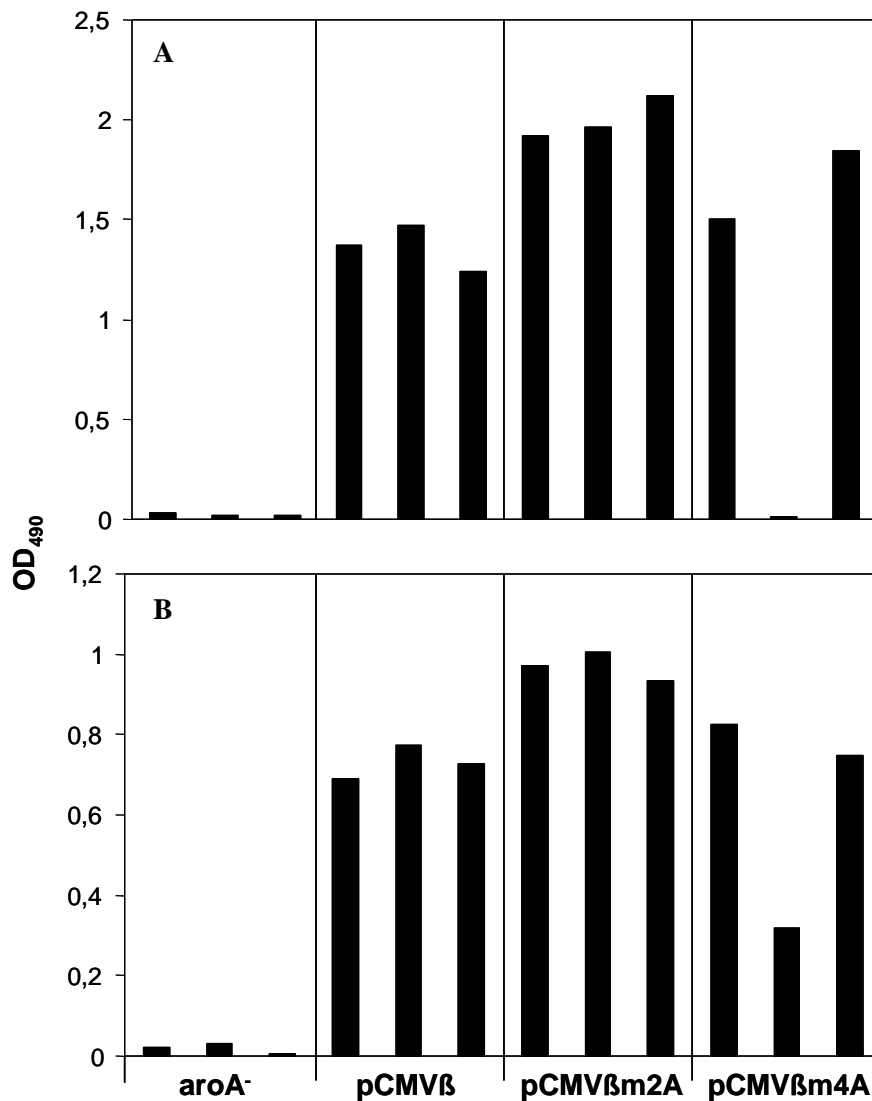


Fig. 4.17: Analysis of antibody response from individual mice. Serum samples from individual mice were analyzed for β -galactosidase-specific antibodies after 4 (A) and 8 (B) weeks p.i. with a single dose of recombinant *Salmonella*.

4.6 Transformation of *S. typhimurium aroA*⁻ with two compatible oris

For the construction of the low copy number plasmids, on the one hand, the pMB1 replicon derived from ColE1 and, on the other hand, the ori from p15A was used. Since these both origins of replication should be compatible in the same bacterium, such a system could prove extremely useful for the co-delivery of antigen and modulating molecules. To show the feasibility of such an approach, *S. typhimurium aroA*⁻ was transformed with the expression plasmid pCMV β m3T together with pCMVhlym2A. Both plasmids could be distinguished in the bacterial cultures due to the different antibiotic resistance marker they carry. Consequently, *Salmonella* carrying these two compatible plasmids should be resistant against ampicillin and tetracycline, thus, facilitating the evaluation of plasmid stability *in vitro* and *in vivo*. Furthermore, the hly gene coding for listeriolysin of *L. monocytogenes* was incorporated into pCMV β m2A replacing β -galactosidase as antigen. Using this second antigen, the induction of immune responses against either of these antigens could be examined after *Salmonella*-mediated gene transfer. In addition, the generation of protective immunity against a lethal challenge with *L. monocytogenes* by the double-transformed *Salmonella* could be analyzed and compared to the strains carrying single low and high copy number vectors encoding listeriolysin.

Stability in vitro and in vivo

First, the plasmid stability of *Salmonella* carrying the two compatible, low copy number vectors pCMV β m3T and pCMVhlym2A was investigated *in vitro*. In the presence of selecting antibiotics, the double-transformed *Salmonella* retain both plasmids at 60% after five passages. Without selection pressure none of the *Salmonella* containing both plasmids could be detected after the second cycle (Fig. 4.18). Plating the *Salmonella* on LB-agar plates containing either tetracycline or ampicillin revealed that only one of the plasmids was lost and the other was retained. No plasmid preference was observed, as depending on the particular experiment one or the other plasmid was lost (data not shown).

These recombinant *Salmonella* were also limited in their ability to colonize murine tissues, since 10- to 100-fold lower number could be detected in the organs compared to pCMV β -carrying *Salmonella*. In addition, only *Salmonella* carrying one

vector, but no double-transformed *Salmonella* could be recovered from the tissues. Again no preference for a particular plasmid was observed, since in one experiment only pCMVhlym2A was recovered, while in the second experiment only pCMV β m3T was retained (data not shown).

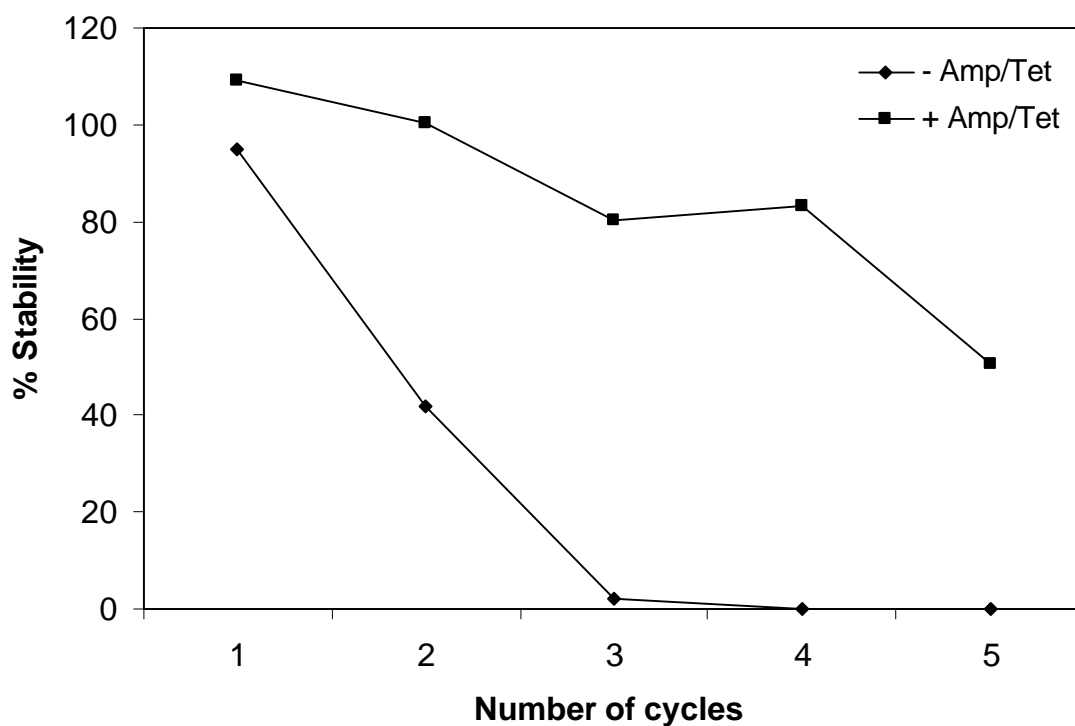


Fig. 4.18: Plasmid stability *in vitro* of *S. typhimurium* *aroA*⁻ carrying two compatible plasmids. *S. typhimurium* was transformed with the low copy vector pCMV β m3T encoding the β -galactosidase as well as with pCMVhlym2A expressing LLO as antigen. Carrying the tetracycline resistance or ampicillin resistance gene, respectively, facilitates selection and assessment of plasmid stability.

Induction of immune responses

Although *Salmonella* carrying two low copy number plasmids with compatible origins of replication were relatively unstable *in vitro* and reduced in their ability to colonize murine tissues, we nevertheless investigated whether this *Salmonella* strain could induce immune responses against both antigens encoded by these compatible plasmids.

Groups of mice received either one or four doses of recombinant *Salmonella* as described above. The ability of the double-transformed *Salmonella* to provoke immune responses was compared to *Salmonella* carrying the high copy plasmid pCMVhly, the low copy number pCMVhlym2A or pCMVβm3T. The mixture of *Salmonella* carrying either pCMVhlym2A or pCMVβm3T at a ratio of 1:1 was included for the single administration and *S. typhimurium aroA*⁻ without plasmid served as negative control.

Helper T cell response

To assess the CD4⁺ helper T cell response, isolated lymphocytes from spleen (Fig. 4.19) and mesenteric lymphnodes (data not shown) were recovered 36 days after a single or the fourth administration of the recombinant bacteria and restimulated either with β-galactosidase or LLO protein as specific antigen (Fig. 4.19A; B). The alternative protein that was not encoded by the immunizing plasmid was used as unspecific control antigen.

Salmonella carrying the compatible plasmids could induce a strong and specific immune response against both antigens after a single administration. Besides, the strength of cell proliferation is comparable to both low copy plasmids given as single construct (Fig. 4.19A). Furthermore, the double-transformed bacteria elicit a CD4⁺ T cell response as strong as a mixture of *Salmonella* carrying either pCMVhlym2A or pCMVβm3T.

Analysis of the CD8⁺ T cell response revealed that approximately the same number of specific IFNγ-producing cells was induced by immunization with *Salmonella* carrying pCMVhlym3T, pCMVβm2A, both plasmids together in the same bacteria, or a mixture of *Salmonella* carrying either of these plasmids (Fig. 14.19C). Multiple applications hardly increased the specific cellular immune responses as observed before (Fig. 4.19B, D).

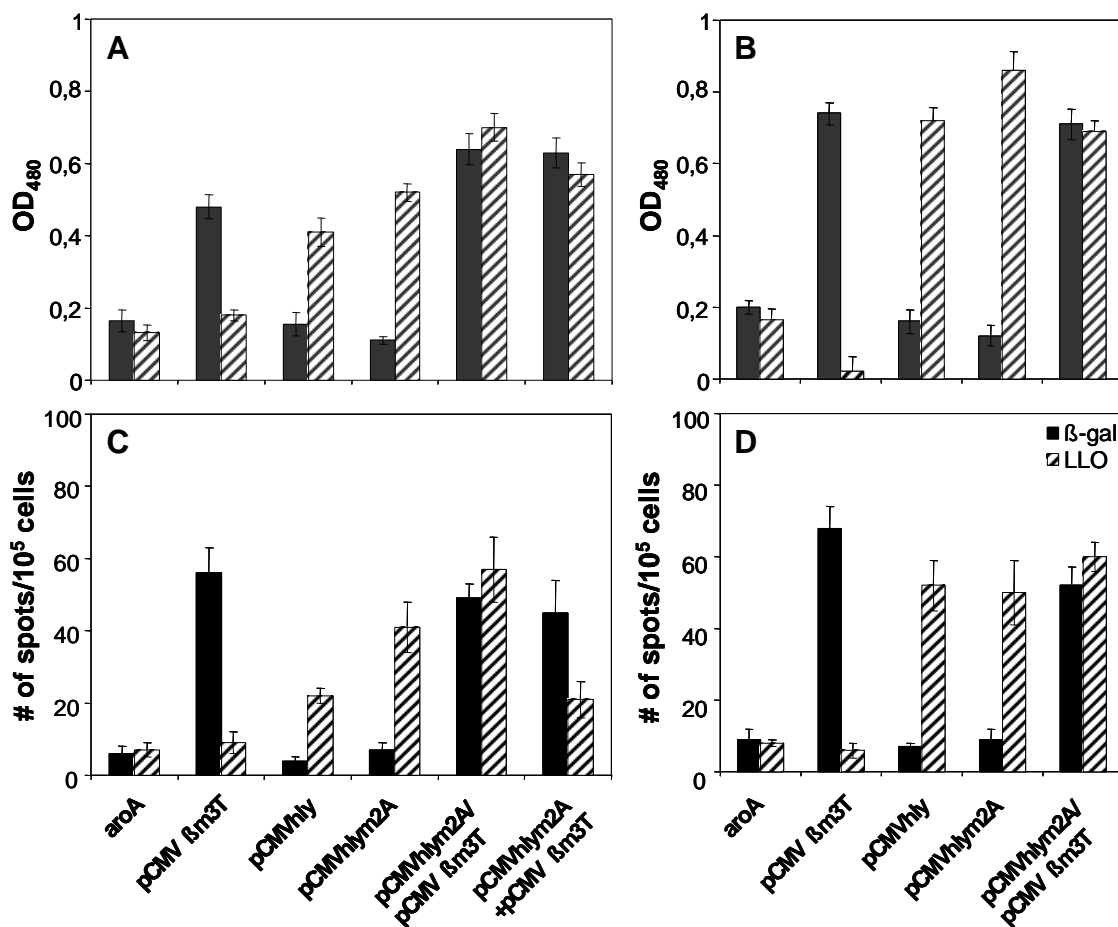


Fig. 4.19: Induction of immune responses in spleen after oral administration of *S. typhimurium* *aroA*⁻ carrying either single or two compatible plasmids. Mice were immunized with *Salmonella* transformed with either the high (pCMVhly) or low copy number plasmid (pCMVhlym2A) encoding listeriolysin (LLO), the low copy number plasmid pCMVβm3T coding for β-galactosidase or the plasmid pCMVhlym2A together with pCMVβm3T (pCMVhlym2A/pCMVβm3T). As negative control, mice received *Salmonella* without plasmid (*aroA*). Immune responses were analyzed 144 days after application of a single (A, C) or 37 days after the fourth (B, D) dose of recombinant *Salmonella*. Antigen specific proliferation of CD4⁺ T cells in response to β-galactosidase or listeriolysin protein was measured in a helper T cell assay and quantitated spectrophotometrically at OD₄₈₀ (A, B). Induction of antigen-specific CD8⁺ T cells was assessed by determining the number of IFNγ-secreting cells after *in vitro* stimulation with β-galactosidase peptide aa 877-885 or LLO peptide aa 91-99, respectively (C, D). In addition, mice were fed orally with a mix of *Salmonella* harboring either pCMVhlym2A or pCMVβm3T (pCMVhlym2A + pCMVβm3T) and immune responses were analysed 36 days after a single administration (A, C).

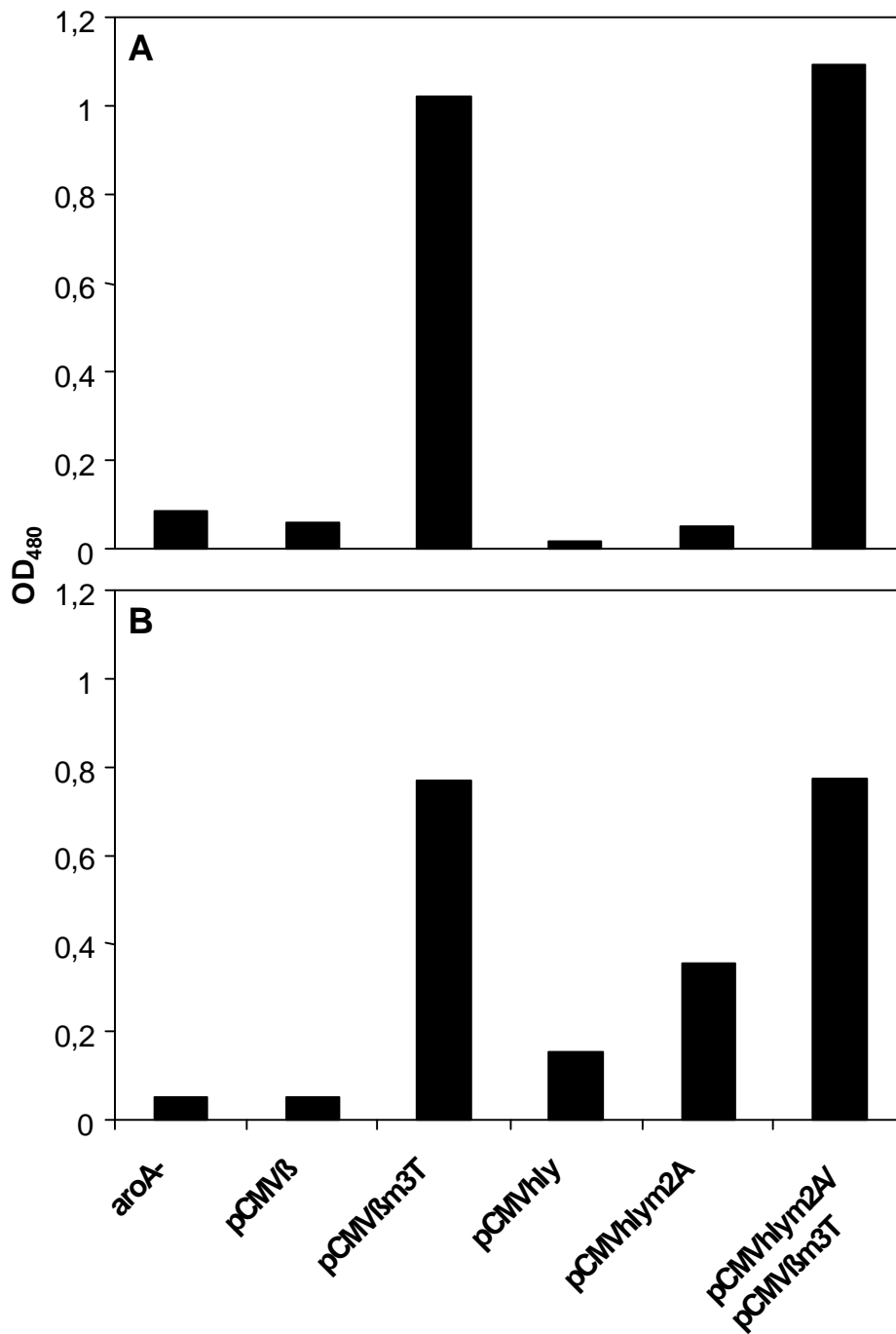


Fig. 4.20: Antibody response against β -galactosidase in BALB/c mice after immunization with *S. typhimurium aroA*⁻ carrying either single or two compatible plasmids. Mice received either a single (A) or four (B) doses of 1×10^8 recombinant *Salmonella* as described above. *Salmonella* without plasmid served as negative control (aroA). Serum samples were assessed for β -gal-specific antibody titers in ELISA in a dilution of 1:1000.

Antibody response

Serum samples of mice immunized once or four times with *Salmonella* carrying the low copy vectors pCMVhlym2A and pCMV β m3T together in the same bacterium were investigated for specific antibodies against listeriolysin and β -galactosidase, respectively. In comparison, *Salmonella* harboring either pCMV β m3T or pCMVhlym2A or a mixture of *Salmonella* strains carrying pCMV β m3T and pCMVhlym2A (data not shown), the double-transformed bacteria stimulated a β -gal-specific antibody response of similar strength. This antibody response could not be increased by multiple administrations of *Salmonella*, as already observed for T cells (Fig. 4.20). The ELISA for specific LLO antibodies, raised by the strains transformed with pCMVhlym2A or pCMVhly, revealed no clear results, indicating low titers.

In summary, immunization with *Salmonella* carrying two different antigens encoded by low copy number vectors with compatible origins of replication, elicits strong and specific immune responses against both, LLO and β -gal. Although these double-transformed *Salmonella* are not as stable as *S. typhimurium* carrying either of the low copy plasmids, the induction of CD4⁺ and CD8⁺ T cells as well as antibodies is comparable and requires only a single administration. In contrast, *Salmonella* harboring the high copy number plasmid that appear to lose their plasmid with a similar kinetic as the double transformed bacteria could stimulate such high responses only when multiple doses were applied.

4.7 Infection of immunized mice with *L. monocytogenes*

To test whether vaccination with *Salmonella* carrying one low copy number vector or two compatible plasmids would result in an immune response that could confer protection against a lethal infection with a pathogenic microorganism, mice were intragastrically inoculated with the recombinant *Salmonella* as above and challenged with a dose of *L. monocytogenes* corresponding to 10 x LD₅₀. As a positive control, mice were immunized with a sublethal dose of 1 x 10³ *L. monocytogenes* 10 days prior to challenge and naïve BALB/c mice served as a negative control. Survival of mice was observed for two weeks post infection.

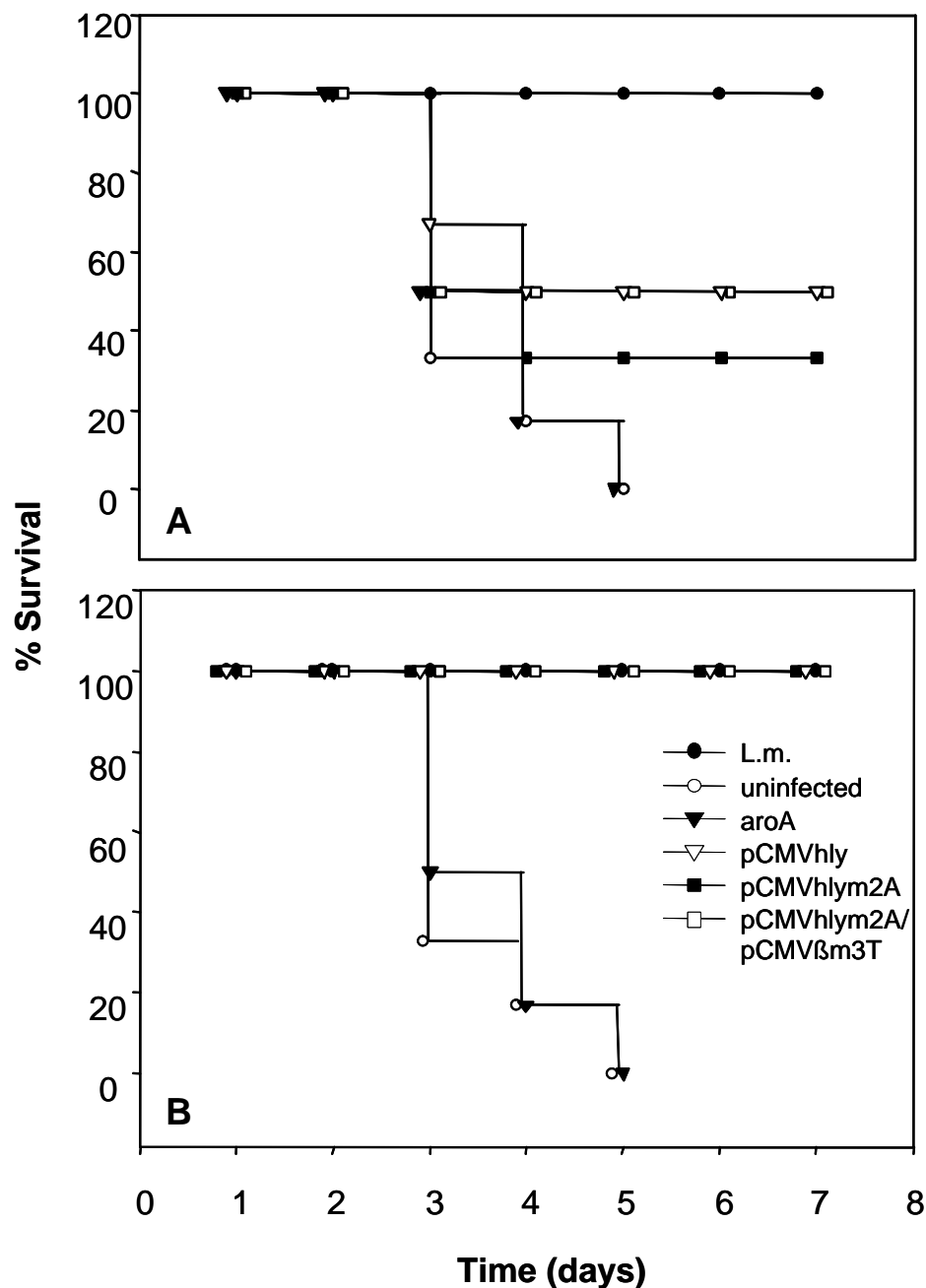


Fig. 4.21: Protection against challenge with a lethal dose of *L. monocytogenes* after oral immunization with various recombinant *S. typhimurium aroA*⁻. BALB/c mice obtained either single (A) or four doses (B) of *Salmonella* carrying the high copy (pCMVhly) or the low copy vector (pCMVhlym2A) expressing LLO, or the low copy vector pCMVhlym2A together with pCMVβm3T (pCMVhlym2A/pCMVβm3T). Naïve mice (uninfected) and mice that received one or four doses of *Salmonella* without plasmid (aroA) served as negative control. As positive control, a group of mice was immunized with a sublethal dose of 1×10^3 *Listeria* (L.m.) 10 days prior to infection.

Mice that had received multiple administrations of transformants harboring a LLO expression plasmid were completely protected, independent of the copy number or a second compatible expression vector (Fig. 4.21B). In contrast, a single administration of such transformants could only partially protect infected BALB/c mice against *L. monocytogenes* (Fig. 4.21A).

All together, lowering the copy number in eukaryotic expression plasmids showed a remarkable increase in plasmid stability *in vitro* as well as *in vivo* and subsequently resulted in an enhanced induction of all specific arms of the immune system. However, in protection studies using listeriolysin as antigen BALB/c mice were only partial protected against a lethal dose of *L. monocytogenes* after a single administration, independent whether *Salmonella* were carrying a high or low copy number vector or two compatible low copy plasmids. Only after multiple applications of LLO plasmid-carrying *Salmonella*, all mice were protected. These observations lead to the conclusion that, despite a stronger induction of immune responses by *Salmonella* transferring low copy number vectors, the generation of memory T cells is not sufficient to protect mice against a lethal dose of *L. monocytogenes* after only a single administration. On the other hand, the new strains of *Salmonella* induced a long-lasting antibody response. Thus, the improved stability can be considered as a step forward in the generation of improved *Salmonella*-based oral DNA vaccines. Employing plasmids with compatible origins of replication as described here will allow further improvements that should finally result in protective immunity after only a single administration.

5 Discussion

Vaccination is the most cost-effective strategy to prevent infectious disease. Despite the introduction of several successful vaccines until today, some major infectious threats remained unchanged and new threats like HIV or drug resistant *Mycobacterium tuberculosis* newly occurred. Thus, there is a growing need to design improved or completely new vaccines. Most vaccines that are currently available are mainly based on the induction of long-lived antibody responses. However, some intracellular bacteria, parasites and viruses also might need T cell-mediated cellular immune responses to be cleared.

In this respect, oral application of DNA vaccines using *Salmonella* as delivery system represents a highly versatile possibility to induce antigen-specific cytotoxic and helper T cell responses, as well as antibodies. This kind of vaccination was far superior to immunization with *Salmonella* expressing the antigen as heterologous protein with regard to immune and memory induction. In addition, mice were partially protected against *L. monocytogenes* when immunized once with the appropriate oral DNA vaccine. Despite the strong induction of cellular immune responses, induction of antibodies is only possible under optimal conditions and mainly systemic instead of mucosal as might have been expected for a mucosally applied vaccine (Darji et al., 2000). Therefore, improvement of *Salmonella*-mediated gene transfer is required to render the system suitable for general application.

It has been reported that strong, constitutive, viral promoters do not cause long-term transgene expression *in vivo* (Scharfmann et al., 1994; Challita and Kohn, 1994; Rettinger et al., 1994), since cytokines like IFN γ and TNF α that are induced during the immune response inhibit transgene expression from viral promoters (Qin et al., 1997). This suggested that the CMV promoter might be not the most appropriate one for *Salmonella*-mediated oral DNA vaccination.

For that reason, *Salmonella*-mediated gene transfer should be improved in this thesis by comparing different eukaryotic promoters with the presently used viral CMV promoter. On the one hand, promoters of housekeeping genes like the elongation factor 1 α promoter or ubiquitin promoter and, on the other hand, tissue specific

promoters like the macrophage specific human lysozyme promoter or the dendritic cell specific CD11c promoter were employed. Such promoters should not be down-regulated by IFN γ . In addition, the promoter of the interferon regulatory factor-1 was tested, since this promoter is activated by IFN γ .

Analysis of CTL responses after immunization with recombinant *Salmonella* revealed that the EF1 α , CD11c and ubiquitin promoter were as good as the CMV promoter in the induction of specific CD8 $^{+}$ T cells. In contrast, stimulation of cytotoxic T cells was extremely low when antigen expression was driven by the hLP or the IRF-1 promoter. Since the hLP promoter is active in macrophages, the weak CD8 $^{+}$ response that was induced after immunization could be explained by the presence of few macrophages in the Peyer's patches. Thus, antigen expression driven by the hLP promoter would have been not sufficient to stimulate a strong CD8 $^{+}$ response. Another possibility for the low response could be that the promoter was weak or not functional at all, as its functionality could not be tested *in vitro* beforehand. Antigen expression driven by the CD11c promoter, surprisingly, induced a strong CTL response. Possibly, antigen expression in the DCs themselves was leading to efficient presentation of antigen and induction of T cells, supporting the important role of DCs as antigen presenting cells after *Salmonella*-mediated gene transfer. In contrast, the immune response provoked by antigen expression from the IRF-1 promoter was unexpectedly low. Either this promoter is very weak, resulting in too low amounts of antigen, or the promoter was not activated by IFN γ . Since IFN γ -secretion by cells of the host defense is induced at rather late time points and all high copy number derivatives of pCMV β are lost rapidly *in vivo* this cytokine might not activate the promoter in time.

This hypothesis would fit well with the observation that constructs containing the CMV promoter elicit strong immune responses, although it was reported that IFN γ down-regulates the activity of the CMV promoter. Consequently, IFN γ production is encountered at a time when the expression plasmid might already be lost. Hence, IFN γ could not inhibit the promoter activity of CMV anymore.

However, such general comparisons between the promoters should be taken cum grano salis, since the experiments have been hampered by severe stability problems of the plasmids in the *Salmonella* carrier *in vitro*. Thus, it became clear that the problem of plasmid stability had to be solved first, before definite conclusions on the optimal promoter could be considered for further improvements of such vaccines.

Accordingly, the main part of this study includes the enhancement of plasmid stability on the basis of different origins of replication to lower the number of copies within the bacterium. To construct different eukaryotic expression plasmids, the conventionally used vector pCMV β was reconstructed in the way that single cassettes for the ori, the resistance gene, the promoter and the antigen coding sequence could be easily exchanged when required. This has the advantage that additional ori's (e.g. F-factor) can be inserted without further manipulation of the plasmid backbone and analyzed comparatively for properties like stability in *Salmonella* as well as their co-existence with other compatible plasmids. In the same way, the promoter and the antigen can be exchanged and investigated for their potential to induce specific immune responses. Finally, antibiotic selection markers could be replaced against different resistance markers, first of all for analysis purposes. Similarly, the antibiotic resistance could be replaced by a balanced suicide system at later stages i.e. a gene that complements a monogenic defect in the bacterial carrier (e.g. *asd*/ Δ *asd*).

After transformation of *S. typhimurium* with the eukaryotic expression plasmids, plasmid stability *in vitro* and *in vivo*, as well as immune responses stimulated after oral immunization, were analyzed. Compared to the high copy number vector pCMV β that bears the pUCori, the low copy number vectors pCMV β m2A, pCMV β m3A and pCMV β m4A containing the pMB1 replicon, p15Aori or pSC101ori, respectively, were all stably retained in the *Salmonella* strain *in vitro* during several passages in LB-broth without any selective pressure. The successful stabilization of plasmids in *Salmonella* without the need of stabilizing antibiotics is especially important for large-scale fermentation. It will simplify the handling and should reduce the costs of production of the vaccine.

In addition, *Salmonella* harboring low copy number expression plasmids could be detected for three weeks in mouse tissues, whereas no *Salmonella* carrying the multicopy plasmid pCMV β could be detected. The increased plasmid stability consequently resulted in a stronger induction of CD4⁺ and CD8⁺ T cells as well as antibody responses already after a single oral administration of 1×10^8 recombinant *S. typhimurium* *aroA*⁻. Furthermore, the strength of immune responses elicited by these low copy number vectors was independent of whether they were carrying the ampicillin or tetracycline resistance cassette.

There exist several reports about immunostimulatory CpG motifs that can enhance the immune response after addition into plasmid DNA. But this property might be only

valuable by using naked plasmid DNA. At least, the exchange of ampicillin, which contains two known CpG motifs, against tetracycline comprising only one known CpG motif in the plasmid backbone had no influence on the induction of immune responses after *Salmonella*-mediated gene transfer. This phenomenon is very likely due to the presence of high amounts of bacterial DNA that overwhelm the influence of minor differences of immuno-stimulatory CpG contents in the plasmid DNA of the eukaryotic expression plasmid by this kind of vaccination.

The lower number of copies present in the *Salmonella* carrier, due to the different origins of replication tested in this study, is responsible for the stable maintenance of the eukaryotic expression plasmids, but also reduces the level of antigen expression. Nevertheless, as shown in this thesis, lower antigen expression over a long period of time by the low copy number vectors pCMV β m2A and pCMV β m3A and their corresponding tetracycline derivatives induce much stronger immune responses after a single application compared to high levels of antigen-expression for a short time by the multicopy plasmid pCMV β . In comparison to the low copy number vectors pCMV β m2A and pCMV β m3A, the induction of immune responses by the very low copy number plasmid pCMV β m4A was weaker and resembled more the high copy number vector pCMV β . In contrast to the original pCMV β plasmid, all low copy number plasmids, including pCMV β m4A, provoked a strong long-lasting antibody response. This suggests that, on the one hand, the stability is more important than the copy number for efficient antigen expression. On the other hand, the copy number should be as high as possible, since the very low copy number plasmid is stably retained in *Salmonella*, but antigen expression due to the very low number of copies was not sufficient to induce exceptionally strong immune responses. Therefore, strategies that enhance antigen expression *in vivo* by these stable low copy plasmids have to be considered. On this score, improvement of DNA vaccine vectors to create *in vivo* amplification systems of antigen expression, such as the alphaviral replicon, might be advantageous. Another approach could make use of a system for the inducible amplification of copy numbers as it is already employed *in vitro* with the pETcoco system (Novagen, Madison, WI). Moreover, the different promoters that were already tested in pUCori-based plasmids should be re-analyzed in the stable low copy plasmids. Such experiments might reveal differences amongst these promoters that were not observable with the unstable high copy number expression plasmids.

Interestingly, induction of long-lasting antibody responses was achieved by the newly but not by the original expression vector system. This might confer protection in infection models where long-lived antibody responses are required for host defense as in the case of extracellular bacteria or viruses. However, despite of this, single administration of the oral *Salmonella*-based DNA vaccine only partially protected mice against *L. monocytogenes* using listeriolysin as antigen in challenge studies. Multiple applications resulted in complete protection against a lethal dose of *L. monocytogenes*. These findings lead to the conclusion that lowering the copy number certainly could enhance the induction of immune responses, but it is not sufficient to induce an adequate T cell memory for protection against an intracellular pathogen like *L. monocytogenes*.

The explanation for incomplete protection via cell-mediated responses might be as follows: Upon primary infection, naïve T cells encounter antigens in the lymphoid tissues and subsequently expand clonally and differentiate into effector T cells. Effector T cells comprise CD4⁺ T cells of the Th1 or Th2 phenotype as well as CD8⁺ T cells known as cytotoxic T lymphocytes. After massive expansion, the majority of effector cells die and immunity contracts to memory levels, during which the number of memory T cells are stabilized and maintained for long periods of time. Thereby, the number of memory T cells is determined by the extent of expansion and cell-death of initial effector cells (reviewed by Kaech et al., 2002). Application of *Salmonella* carrying low copy number expression plasmids obviously results in an increased recruitment or proliferation, since stronger T cell responses were observed. However, these improved vaccines seem not to interfere with the subsequent contraction phase. Therefore, the identification of molecules that interfere with cell-death might enhance T-cell memory and in turn, might provide better immunological memory after a single vaccination.

So far, several cytokines and co-stimulatory molecules are known, which influence the quantity and quality of memory T cells. Cytokines, like type I IFNs and members of the IL-2 family (IL-2, IL-4, IL-7 and IL-15), were shown to reduce the rate of cell-death *in vitro* and in some cases *in vivo* by directly inhibiting apoptosis and/or enhancing effector T cell proliferation (Zhang et al., 1998; Tan et al., 2001; Vella et al., 1998; Marrack et al., 1999; Sprent et al., 2000). Moreover, it was demonstrated that high levels of IL-4, IL-7 and IL-15 *in vivo* could increase the number of antigen-specific CD4⁺ and CD8⁺ T cells and IL-15 could additionally enhance protective

responses (Vella et al., 1998; Yajima et al., 2002; Khan and Casciotti, 1999; Maeurer et al., 2000).

Similarly, molecules of the TNF receptor family and their ligands like CD27 and CD154 (CD40L) might also play an important role in the development of memory T cells. As example, experiments with CD154-deficient mice revealed that CD40-CD154 interactions interfere with the contraction phase and prevent death of effector CD8⁺ T cells, consequently influencing the formation of memory T cells (Borrow et al., 1996; Whitmire and Ahmed, 2000). In addition, CD154 is essential for the induction of T cell-mediated protective immunity against viruses, parasites and tumors (Gurunathan et al., 1998; Sin et al., 2001; Xiang et al., 2001) by providing costimulatory activity on DCs via CD40-CD154 interactions. Co-administration of one or more of such molecules together with specific antigen might improve the *Salmonella*-mediated gene-transfer in such a way that protective immunity is achieved already after a single oral administration.

Moreover, co-delivery of different costimulatory molecules or cytokines that can modulate the immune responses into the desired direction might likewise confer better protection. For instance, co-expression of IL-4 or IFN γ and IL-12 could bias T cells into a Th2 type or Th1 type response, respectively. Thereby, these cytokines might support appropriate effector functions and help to clear pathogens.

Co-delivery of modulating molecules can be achieved by bi- or multicistronic expression using an IRES sequence. But encoding two or more genes on the same plasmid enlarges the plasmid size and thus might influence plasmid stability. In addition, gene-co-expression behind an IRES is often diminished and sometimes even not possible. In this respect, the use of plasmids with compatible ori's in the same bacterium as described in this thesis could solve such problems. It was already shown in this study that two unrelated antigens encoded on compatible plasmids could generate strong CD4⁺ as well as CD8⁺ T cell responses against both antigens. Within the time frame of this thesis, it was not possible to definitely demonstrate, whether both antigens are expressed in the same target cell. This might be important in cases of immune modulation or where the homing of APCs should be influenced e.g. by the expression of chemokine receptors or integrins, which target APCs to gut associated mucosal tissues. Nevertheless, compatible plasmids should result in further improvements that finally lead to protective immunity upon a single application of *Salmonella*-based oral DNA vaccines.

In the same way, the selection of the *Salmonella* carrier (as already mentioned above) might have an impact on the outcome of protective immunity. Whereas *S. typhimurium aroA⁻* requires Th1 cells and IFN γ for clearance, *S. typhimurium* of the PhoP⁻ phenotype involves innate immune responses of macrophages (Van Cott, 1998). Consequently, more systematic experiments will be required to find an optimal combination of features of the expression plasmid and the appropriate bacterial carrier.

Taken together, it was demonstrated that lowering the copy number strongly increase plasmid stability *in vitro* and *in vivo* and consequently enhance specific immune responses after oral DNA vaccination with *S. typhimurium aroA⁻*. Complete protection against a lethal challenge with *L. monocytogenes* after application of the low copy number vectors as well as with the multicopy plasmid pCMV β was only possible after multiple vaccinations. This indicates that a single dose is not sufficient to recruit a strong immunological memory for cellular mediated responses as required for clearance of intracellular pathogens like *L. monocytogenes*. However, *Salmonella* bearing low copy number expression plasmids induce exceptionally long-lived specific antibodies that have been never observed before using the original high copy number plasmid pCMV β (Darji et al., 2000), after already a single administration. Hence, using the appropriate infectious model e.g. a virus model that need a strong antibody mediated response to resolve the infection, mice might be protected even after a single oral dose.

Thus, we consider the improved stability as a first step towards a second generation of *Salmonella*-based oral DNA vaccines. The employment of plasmids with compatible origins, as described in this study, will allow further improvements that will confer protective immunity already after a single oral dose of recombinant *Salmonella* vaccines. More systematic analysis of this vaccination strategy will pave the way for the development of efficient vaccines that are general applicable to treat infectious diseases of bacteria, parasites and viruses as well as cancer and allergic diseases.

6 Summary

Salmonella-mediated oral DNA vaccination has established itself as a very potent and versatile way of immunization. The use of commercially available multicopy expression vectors based on pUC origin of replication (ori) transferred by *Salmonella typhimurium aroA*⁻ to mice has resulted in protective responses against pathogens and tumors. Nevertheless, the present combination of the *Salmonella* carrier and the antigen-expressing plasmid could induce strong antibody responses only under optimal conditions (Darji et al., 1997; 2000). In addition, to protect mice against a lethal dose of *Listeria monocytogenes*, several administrations of *Salmonella* carrying listeriolysin as antigen had to be applied. Therefore, an improved expression vector system should be developed. First, high copy number expression plasmids with different eukaryotic promoters were tested. No improvement over the original CMV promoter was observed. However, during these experiments, it was recognized that the general applicability of this system has been hampered by a severe instability of the expression plasmids.

Thus, new low copy number expression plasmids were constructed using different ori's to stabilize the plasmid. Comparative studies between the high copy number plasmid pCMV β and the different low copy number plasmids, carrying the pMB1, p15A or pSC101 ori on the pCMV β backbone, revealed a dramatic increase in plasmid stability *in vitro* as well as *in vivo*. Analyzing the immune responses against antigens encoded by these vectors indicated that the increased stability consequently resulted in a stronger induction of antigen specific CD4⁺ and CD8⁺ T cell as well as antibody responses, already after a single administration. In protection studies using listeriolysin as antigen, mice were only partial protected against a lethal dose of *L. monocytogenes* after a single administration, independent of whether *Salmonella* were carrying the high or the low copy number plasmids. After four immunizations, all mice were completely protected. However, in contrast to immunization with *Salmonella* bearing the high copy number plasmid, the newly established *Salmonella* harboring low copy number expression plasmids induce a long-lasting antibody

response. Thus, the improved stability of the vectors represents a first step towards a second generation of *Salmonella*-based oral DNA vaccines.

Low copy number compatible ori's allow transformation of *Salmonella* with two expression plasmids. Such *Salmonella* strain confers strong immunity to the different plasmid derived antigens, although the plasmids were as stable as single low copy vectors alone. Nevertheless, this strategy will allow further improvements e.g. by coadministration of plasmids encoding cytokines or costimulatory molecules. Such improvements will finally lead to the designated goal of establishing an oral DNA vaccine that yields a live long protective response already after a single administration.

7 Abbreviations

aa	amino acid
AmpR	ampicillin resistance marker
APC	antigen presenting cell
asd	aspartate-semialdehyddehydrogenase
BHI	<u>b</u> rain <u>h</u> eart <u>i</u> nfusion
bp	baisepairs
CD	cluster of differentiation
cfu	colony forming units
CmR	chloramphenicol resistance gene
CMV	cytomegalovirus
CpG	<u>C</u> ytosine- <u>p</u> hosphate- <u>G</u> uanosine
CTB	<u>c</u> holera <u>t</u> oxin subunit <u>B</u>
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DHB	2, 3-dihydroxybenzoate
DNA	deoxyribonucleic acid
DPT	<u>D</u> iphtheria <u>P</u> ertussis <u>T</u> oxoids
E:T	effector-to-target ratio
EDTA	ethylenediaminetetraacetate
ELISA	<u>e</u> nzyme- <u>l</u> inked <u>i</u> mmuno <u>s</u> orbent <u>a</u> ssay
ELISPOT	<u>e</u> nzyme- <u>l</u> inked <u>i</u> mmuno <u>s</u> pot assay
F	Farad
FAE	follicle-associated epithelium
FCS	<u>f</u> etal <u>c</u> alf <u>s</u> erum
g	gravitational acceleration
GM-CSF	<u>g</u> ranulocyte <u>m</u> acrophage- <u>c</u> olony <u>s</u> timulatory <u>f</u> actor
gp	glycoprotein
HIV	<u>H</u> uman <u>I</u> mmunodeficiency <u>V</u> irus
HLA	<u>h</u> uman <u>l</u> eukocyte <u>a</u> ntigenes

HSV	<u>h</u> uman <u>s</u> imian <u>v</u> irus
i.v.	intravenous
IFN	Interferon
Ig	immunoglobulin
IL	Interleukin
IMDM	Iscoe's modified Dulbecco's modified Eagle's medium
IRES	<u>i</u> nternal <u>r</u> ibosomal <u>e</u> ntry <u>s</u> ite
IRF	<u>i</u> nterferon <u>r</u> egulatory <u>f</u> actor
ISCOM	<u>i</u> mmune <u>s</u> timulatory <u>c</u> omplexes
kanR	kanamycin resistance gene
LACK	<u>L</u> eishmania homologue of receptors for <u>a</u> ctivated <u>C</u> <u>k</u> inase
LB	<u>L</u> uria <u>B</u> ertani
LLO	<u>l</u> isteriolysin <u>O</u>
LN	lymphnode
LTB	B-subunit of the heat-labile enterotoxin from <i>E. coli</i>
M cell	microfold cell
MALT	<u>m</u> ucosa- <u>a</u> ssociated <u>l</u> ymphoid <u>t</u> issue
MHC	<u>m</u> ajor <u>h</u> istocompatibility <u>c</u> omplex
MOI	<u>m</u> ultiplicity <u>o</u> f <u>i</u> nfection
NK cell	natural killer cell
OD	<u>o</u> ptical <u>d</u> ensity
OPD	<u>O</u> - <u>p</u> henylend <u>a</u> mine
ori	origin of replication
p.i.	post infection
pAB	p-aminobenzoid acid
PBS	phosphate buffered saline
PCR	<u>p</u> olymearase <u>c</u> hain <u>r</u> eaction
PEG	polyethylenglycol
PP	Peyer's patches
PrV gD	glycoprotein D gene of pseudorabies virus
rpm	rounds per minute
RT	room temperature
spp.	subspecies
TAE	tris-acetic acid-EDTA

TE	trypsin-EDTA
TetR	tetracycline resistance gene
Th cell	<u>T</u> <u>h</u> elper cell
TLR	<u>T</u> oll- <u>l</u> ike <u>r</u> eceptor
Tn	transposon
TNF	<u>t</u> umor <u>n</u> ecrosis <u>f</u> actor
U	unit
UV	ultraviolet
V	Volt
v/v	volume per volume
w/v	weight per volume

8 References

- Acheson,D.W., Levine,M.M., Kaper,J.B., and Keusch,G.T. (1996). Protective immunity to Shiga-like toxin I following oral immunization with Shiga-like toxin I B-subunit-producing *Vibrio cholerae* CVD 103-HgR. *Infect. Immun.* 64, 355-357.
- Ada, G.L.: The immunological principles of vaccination. *Lancet* 1990, 335:523-526.
- Aderem,A. and Ulevitch,R.J. (2000). Toll-like receptors in the induction of the innate immune response. *Nature* 406, 782-787.
- Aggarwal,A., Kumar,S., Jaffe,R., Hone,D., Gross,M., and Sadoff,J. (1990). Oral Salmonella: malaria circumsporozoite recombinants induce specific CD8+ cytotoxic T cells. *J. Exp. Med.* 172, 1083-1090.
- Akbari,O., Panjwani,N., Garcia,S., Tascon,R., Lowrie,D., and Stockinger,B. (1999). DNA vaccination: transfection and activation of dendritic cells as key events for immunity. *J. Exp. Med.* 189, 169-178.
- Al Mariri,A., Tibor,A., Lestrade,P., Mertens,P., De,B., X, and Letesson,J.J. (2002). *Yersinia enterocolitica* as a vehicle for a naked DNA vaccine encoding *Brucella abortus* bacterioferritin or P39 antigen. *Infect. Immun.* 70, 1915-1923.
- Amara,R.R., Villinger,F., Altman,J.D., Lydy,S.L., O'Neil,S.P., Staprans,S.I., Montefiori,D.C., Xu,Y., Herndon,J.G., Wyatt,L.S., Candido,M.A., Kozyr,N.L., Earl,P.L., Smith,J.M., Ma,H.L., Grimm,B.D., Hulse,M.L., Miller,J., McClure,H.M., McNicholl,J.M., Moss,B., and Robinson,H.L. (2001). Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. *Science* 292, 69-74.
- Andre,S., Seed,B., Eberle,J., Schraut,W., Bultmann,A., and Haas,J. (1998). Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage. *J. Virol.* 72, 1497-1503.
- Arakawa,T., Chong,D.K., and Langridge,W.H. (1998). Efficacy of a food plant-based oral cholera toxin B subunit vaccine. *Nat. Biotechnol.* 16, 292-297.
- Bacon,G.A., Burrows,T.W., and Yates,M. (1950). The effects of biochemical mutations on the virulence of *Bacterium typhosum*; the virulence of mutants. *Br. J. Exp. Pathol.* 31, 714-724.
- Bacon,G.A., Burrows,T.W., and Yates,M. (1951). The effects of biochemical mutations on the virulence of *Bacterium typhosum*. The loss of virulence of certain mutants. *Br. J. Exp. Path.* 32, 85-96.
- Barrow,P.A., Hassan,J.O., Lovell,M.A., and Berchieri,A. (1990). Vaccination of chickens with *aroA* and other mutants of *Salmonella typhimurium* and *S. enteritidis*. *Res. Microbiol.* 141, 851-853.

- Bendigs,S., Salzer,U., Lipford,G.B., Wagner,H., and Heeg,K. (1999). CpG-oligodeoxynucleotides co-stimulate primary T cells in the absence of antigen-presenting cells. *Eur. J. Immunol.* 29, 1209-1218.
- Berglund,P., Tubulekas,I., and Liljestrom,P. (1996). Alphaviruses as vectors for gene delivery. *Trends Biotechnol.* 14, 130-134.
- Berglund,P., Smerdou,C., Fleeton,M.N., Tubulekas,I., and Liljestrom,P. (1998). Enhancing immune responses using suicidal DNA vaccines. *Nat. Biotechnol.* 16, 562-565.
- Bliska,J.B., Galan,J.E., and Falkow,S. (1993). Signal transduction in the mammalian cell during bacterial attachment and entry. *Cell* 73, 903-920.
- Borrow,P., Tishon,A., Lee,S., Xu,J., Grewal,I.S., Oldstone,M.B., and Flavell,R.A. (1996). CD40L-deficient mice show deficits in antiviral immunity and have an impaired memory CD8+ CTL response. *J. Exp. Med.* 183, 2129-2142.
- Boshart,M., Weber,F., Jahn,G., Dorsch-Hasler,K., Fleckenstein,B., and Schaffner,W. (1985). A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell* 41, 521-530.
- Boyer,J.D., Chattergoon,M., Shah,A., Ginsberg,R., MacGregor,R.R., and Weiner,D.B. (1998). HIV-1 DNA based vaccine induces a CD8 mediated cross-clade CTL response. *Dev. Biol. Stand.* 95, 147-153.
- Brennan,M.A. and Cookson,B.T. (2000). Salmonella induces macrophage death by caspase-1-dependent necrosis. *Mol. Microbiol.* 38, 31-40.
- Brunham,R.C. and Zhang,D. (1999). Transgene as vaccine for chlamydia. *Am. Heart J.* 138, S519-S522.
- Cardenas,L. and Clements,J.D. (1993). Development of mucosal protection against the heat-stable enterotoxin (ST) of *Escherichia coli* by oral immunization with a genetic fusion delivered by a bacterial vector. *Infect. Immun.* 61, 4629-4636.
- Carter,P.B. and Collins,F.M. (1974). The route of enteric infection in normal mice. *J. Exp. Med.* 139, 1189-1203.
- Chabalgoity,J.A., Khan,C.M., Nash,A.A., and Hormaeche,C.E. (1996). A *Salmonella typhimurium* htrA live vaccine expressing multiple copies of a peptide comprising amino acids 8-23 of herpes simplex virus glycoprotein D as a genetic fusion to tetanus toxin fragment C protects mice from herpes simplex virus infection. *Mol. Microbiol.* 19, 791-801.
- Challita,P.M. and Kohn,D.B. (1994). Lack of expression from a retroviral vector after transduction of murine hematopoietic stem cells is associated with methylation in vivo. *Proc. Natl. Acad. Sci. U. S A* 91, 2567-2571.

- Chang,A.C. and Cohen,S.N. (1978). Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J. Bacteriol.* 134, 1141-1156.
- Chapman,B.S., Thayer,R.M., Vincent,K.A., and Haigwood,N.L. (1991). Effect of intron A from human cytomegalovirus (Towne) immediate-early gene on heterologous expression in mammalian cells. *Nucleic Acids Res.* 19, 3979-3986.
- Charbit,A., Martineau,P., Ronco,J., Leclerc,C., Lo-Man,R., Michel,V., O'Callaghan,D., and Hofnung,M. (1993). Expression and immunogenicity of the V3 loop from the envelope of human immunodeficiency virus type 1 in an attenuated *aroA* strain of *Salmonella typhimurium* upon genetic coupling to two *Escherichia coli* carrier proteins. *Vaccine* 11, 1221-1228.
- Charpentier,E., Gerbaud,G., and Courvalin,P. (1999). Conjugative mobilization of the rolling-circle plasmid pIP823 from *Listeria monocytogenes* BM4293 among gram-positive and gram-negative bacteria. *J. Bacteriol.* 181, 3368-3374.
- Chatfield,S., Roberts,M., Li,J., Starns,A., and Dougan,G. (1994). The use of live attenuated *Salmonella* for oral vaccination. *Dev. Biol. Stand.* 82, 35-42.
- Chatfield,S.N., Charles,I.G., Makoff,A.J., Oxeer,M.D., Dougan,G., Pickard,D., Slater,D., and Fairweather,N.F. (1992a). Use of the *nirB* promoter to direct the stable expression of heterologous antigens in *Salmonella* oral vaccine strains: development of a single-dose oral tetanus vaccine. *Biotechnology (N. Y.)* 10, 888-892.
- Chatfield,S.N., Strahan,K., Pickard,D., Charles,I.G., Hormaeche,C.E., and Dougan,G. (1992b). Evaluation of *Salmonella typhimurium* strains harbouring defined mutations in *htrA* and *aroA* in the murine salmonellosis model. *Microb. Pathog.* 12, 145-151.
- Chatfield,S.N., Roberts,M., Dougan,G., Hormaeche,C., and Khan,C.M. (1995). The development of oral vaccines against parasitic diseases utilizing live attenuated *Salmonella*. *Parasitology* 110 Suppl, S17-S24.
- Chen,L.M., Kaniga,K., and Galan,J.E. (1996). *Salmonella* spp. are cytotoxic for cultured macrophages. *Mol. Microbiol.* 21, 1101-1115.
- Cheng,L., Ziegelhoffer,P.R., and Yang,N.S. (1993). In vivo promoter activity and transgene expression in mammalian somatic tissues evaluated by using particle bombardment. *Proc. Natl. Acad. Sci. U. S A* 90, 4455-4459.
- Clark,M.A., Jepson,M.A., Simmons,N.L., and Hirst,B.H. (1994). Preferential interaction of *Salmonella typhimurium* with mouse Peyer's patch M cells. *Res. Microbiol.* 145, 543-552.
- Clarke,S., Greaves,D.R., Chung,L.P., Tree,P., and Gordon,S. (1996). The human lysozyme promoter directs reporter gene expression to activated myelomonocytic cells in transgenic mice. *Proc. Natl. Acad. Sci. U. S A* 93, 1434-1438.

- Clements,J.D., Lyon,F.L., Lowe,K.L., Farrand,A.L., and el Morshidy,S. (1986). Oral immunization of mice with attenuated *Salmonella enteritidis* containing a recombinant plasmid which codes for production of the B subunit of heat-labile *Escherichia coli* enterotoxin. *Infect. Immun.* 53, 685-692.
- Collins,F.M. (1974). Vaccines and cell-mediated immunity. *Bacteriol. Rev.* 38, 371-402.
- Condon,C., Watkins,S.C., Celluzzi,C.M., Thompson,K., and Falo,L.D., Jr. (1996). DNA-based immunization by in vivo transfection of dendritic cells. *Nat. Med.* 2, 1122-1128.
- Cookson,B.T. and Brennan,M.A. (2001). Pro-inflammatory programmed cell death. *Trends Microbiol.* 9, 113-114.
- Cooper,G.L., Nicholas,R.A., Cullen,G.A., and Hormaeche,C.E. (1990). Vaccination of chickens with a *Salmonella enteritidis* aroA live oral *Salmonella* vaccine. *Microb. Pathog.* 9, 255-265.
- Corr,M., Lee,D.J., Carson,D.A., and Tighe,H. (1996). Gene vaccination with naked plasmid DNA: mechanism of CTL priming. *J. Exp. Med.* 184, 1555-1560.
- Coulson,N.M., Fulop,M., and Titball,R.W. (1994). Effect of different plasmids on colonization of mouse tissues by the aromatic amino acid dependent *Salmonella typhimurium* SL 3261. *Microb. Pathog.* 16, 305-311.
- Courvalin,P., Goussard,S., and Grillot-Courvalin,C. (1995). Gene transfer from bacteria to mammalian cells. *C R. Acad. Sci. III* 318, 1207-1212.
- Cox,J.C., Sjolander,A., and Barr,I.G. (1998). ISCOMs and other saponin based adjuvants. *Adv. Drug Deliv. Rev.* 32, 247-271.
- Cunningham,C. and Nemunaitis,J. (2001). A phase I trial of genetically modified *Salmonella typhimurium* expressing cytosine deaminase (TAPET-CD, VNP20029) administered by intratumoral injection in combination with 5-fluorocytosine for patients with advanced or metastatic cancer. Protocol no: CL-017. Version: April 9, 2001. *Hum. Gene Ther.* 12, 1594-1596.
- Curtiss,R., III and Kelly,S.M. (1987). *Salmonella typhimurium* deletion mutants lacking adenylate cyclase and cyclic AMP receptor protein are avirulent and immunogenic. *Infect. Immun.* 55, 3035-3043.
- Curtiss,R., III, Galan,J.E., Nakayama,K., and Kelly,S.M. (1990). Stabilization of recombinant avirulent vaccine strains in vivo. *Res. Microbiol.* 141, 797-805.
- Darji,A., Chakraborty,T., Niebuhr,K., Tsonis,N., Wehland,J., and Weiss,S. (1995). Hyperexpression of listeriolysin in the nonpathogenic species *Listeria innocua* and high yield purification. *J. Biotechnol.* 43, 205-212.

- Darji,A., Guzman,C.A., Gerstel,B., Wachholz,P., Timmis,K.N., Wehland,J., Chakraborty,T., and Weiss,S. (1997). Oral somatic transgene vaccination using attenuated *S. typhimurium*. *Cell* 91, 765-775.
- Darji,A., zur,L.S., Garbe,A.I., Chakraborty,T., and Weiss,S. (2000). Oral delivery of DNA vaccines using attenuated *Salmonella typhimurium* as carrier. *FEMS Immunol. Med. Microbiol.* 27, 341-349.
- Davis,H.L., Michel,M.L., Mancini,M., Schleef,M., and Whalen,R.G. (1994). Direct gene transfer in skeletal muscle: plasmid DNA-based immunization against the hepatitis B virus surface antigen. *Vaccine* 12, 1503-1509.
- Dietrich,G., Bubert,A., Gentschev,I., Sokolovic,Z., Simm,A., Catic,A., Kaufmann,S.H., Hess,J., Szalay,A.A., and Goebel,W. (1998). Delivery of antigen-encoding plasmid DNA into the cytosol of macrophages by attenuated suicide *Listeria monocytogenes*. *Nat. Biotechnol.* 16, 181-185.
- Dietrich,G., Spreng,S., Gentschev,I., and Goebel,W. (2000). Bacterial systems for the delivery of eukaryotic antigen expression vectors. *Antisense Nucleic Acid Drug Dev.* 10, 391-399.
- Dietrich,G., Kolb-Maurer,A., Spreng,S., Scharl,M., Goebel,W., and Gentschev,I. (2001). Gram-positive and Gram-negative bacteria as carrier systems for DNA vaccines. *Vaccine* 19, 2506-2512.
- Dighe,A.S., Campbell,D., Hsieh,C.S., Clarke,S., Greaves,D.R., Gordon,S., Murphy,K.M., and Schreiber,R.D. (1995). Tissue-specific targeting of cytokine unresponsiveness in transgenic mice. *Immunity* 3, 657-666.
- Doe,B., Selby,M., Barnett,S., Baenziger,J., and Walker,C.M. (1996). Induction of cytotoxic T lymphocytes by intramuscular immunization with plasmid DNA is facilitated by bone marrow-derived cells. *Proc. Natl. Acad. Sci. U. S A* 93, 8578-8583.
- Donnelly,J.J., Friedman,A., Martinez,D., Montgomery,D.L., Shiver,J.W., Motzel,S.L., Ulmer,J.B., and Liu,M.A. (1995). Preclinical efficacy of a prototype DNA vaccine: enhanced protection against antigenic drift in influenza virus. *Nat. Med.* 1, 583-587.
- Donnelly,J.J., Ulmer,J.B., Shiver,J.W., and Liu,M.A. (1997). DNA vaccines. *Annu. Rev. Immunol.* 15, 617-648.
- Dougan,G., Chatfield,S., Pickard,D., Bester,J., O'Callaghan,D., and Maskell,D. (1988). Construction and characterization of vaccine strains of *Salmonella* harboring mutations in two different *aro* genes. *J. Infect. Dis.* 158, 1329-1335.
- Dougan,G. (1994). 1993 Colworth Prize Lecture. The molecular basis for the virulence of bacterial pathogens: implications for oral vaccine development. *Microbiology* 140 (Pt 2), 215-224.

- Dunstan,S.J., Simmons,C.P., and Strugnell,R.A. (1998). Comparison of the abilities of different attenuated *Salmonella typhimurium* strains to elicit humoral immune responses against a heterologous antigen. *Infect. Immun.* 66, 732-740.
- Eldridge,J.H., Staas,J.K., Meulbroek,J.A., Tice,T.R., and Gilley,R.M. (1991). Biodegradable and biocompatible poly(DL-lactide-co-glycolide) microspheres as an adjuvant for staphylococcal enterotoxin B toxoid which enhances the level of toxin-neutralizing antibodies. *Infect. Immun.* 59, 2978-2986.
- Eo,S.K., Gierynska,M., Kamar,A.A., and Rouse,B.T. (2001). Prime-boost immunization with DNA vaccine: mucosal route of administration changes the rules. *J. Immunol.* 166, 5473-5479.
- Ertl,H.C. and Xiang,Z. (1996). Novel vaccine approaches. *J. Immunol.* 156, 3579-3582.
- Fairweather,N.F., Chatfield,S.N., Makoff,A.J., Strugnell,R.A., Bester,J., Maskell,D.J., and Dougan,G. (1990). Oral vaccination of mice against tetanus by use of a live attenuated *Salmonella* carrier. *Infect. Immun.* 58, 1323-1326.
- Feltquate,D.M., Heaney,S., Webster,R.G., and Robinson,H.L. (1997). Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. *J. Immunol.* 158, 2278-2284.
- Fennelly,G.J., Khan,S.A., Abadi,M.A., Wild,T.F., and Bloom,B.R. (1999). Mucosal DNA vaccine immunization against measles with a highly attenuated *Shigella flexneri* vector. *J. Immunol.* 162, 1603-1610.
- Finlay,B.B., Ruschkowski,S., and Dedhar,S. (1991). Cytoskeletal rearrangements accompanying salmonella entry into epithelial cells. *J. Cell Sci.* 99 (Pt 2), 283-296.
- Flo,J., Tisminetzky,S., and Baralle,F. (2001). Oral transgene vaccination mediated by attenuated *Salmonellae* is an effective method to prevent Herpes simplex virus-2 induced disease in mice. *Vaccine* 19, 1772-1782.
- Fooks,A.R., Schadeck,E., Liebert,U.G., Dowsett,A.B., Rima,B.K., Steward,M., Stephenson,J.R., and Wilkinson,G.W. (1995). High-level expression of the measles virus nucleocapsid protein by using a replication-deficient adenovirus vector: induction of an MHC-1-restricted CTL response and protection in a murine model. *Virology* 210, 456-465.
- Fouts,T.R., Tuskan,R.G., Chada,S., Hone,D.M., and Lewis,G.K. (1995). Construction and immunogenicity of *Salmonella typhimurium* vaccine vectors that express HIV-1 gp120. *Vaccine* 13, 1697-1705.
- Fox,B.C., Roof,M.B., Carter,D.P., Kesl,L.D., and Roth,J.A. (1997). Safety and efficacy of an avirulent live *Salmonella choleraesuis* vaccine for protection of calves against S dublin infection. *Am. J. Vet. Res.* 58, 265-271.

- Francis, C.L., Starnbach, M.N., and Falkow, S. (1992). Morphological and cytoskeletal changes in epithelial cells occur immediately upon interaction with *Salmonella typhimurium* grown under low-oxygen conditions. *Mol. Microbiol.* 6, 3077-3087.
- Francis, C.L., Ryan, T.A., Jones, B.D., Smith, S.J., and Falkow, S. (1993). Ruffles induced by *Salmonella* and other stimuli direct macropinocytosis of bacteria. *Nature* 364, 639-642.
- Frey, A., Giannasca, K.T., Weltzin, R., Giannasca, P.J., Reggio, H., Lencer, W.I., and Neutra, M.R. (1996). Role of the glycocalyx in regulating access of microparticles to apical plasma membranes of intestinal epithelial cells: implications for microbial attachment and oral vaccine targeting. *J. Exp. Med.* 184, 1045-1059.
- Fu, T.M., Friedman, A., Ulmer, J.B., Liu, M.A., and Donnelly, J.J. (1997). Protective cellular immunity: cytotoxic T-lymphocyte responses against dominant and recessive epitopes of influenza virus nucleoprotein induced by DNA immunization. *J. Virol.* 71, 2715-2721.
- Fuller, D.H., Corb, M.M., Barnett, S., Steimer, K., and Haynes, J.R. (1997). Enhancement of immunodeficiency virus-specific immune responses in DNA-immunized rhesus macaques. *Vaccine* 15, 924-926.
- Galan, J.E. and Collmer, A. (1999). Type III secretion machines: bacterial devices for protein delivery into host cells. *Science* 284, 1322-1328.
- Garmory, H.S., Brown, K.A., and Titball, R.W. (2002a). *Salmonella* vaccines for use in humans: present and future perspectives. *FEMS Microbiol. Rev.* 26, 339-353.
- Garmory, H.S., Griffin, K.F., Leary, S.E., Perkins, S.D., Brown, K.A., and Titball, R.W. (2002b). The effect of recombinant plasmids on in vivo colonisation of *Salmonella enterica* serovar Typhimurium strains is not reflected by in vitro cellular invasion assays. *Vaccine* 20, 3239-3243.
- Garmory, H.S., Griffin, K.F., Brown, K.A., and Titball, R.W. (2003). Oral immunisation with live *aroA* attenuated *Salmonella enterica* serovar Typhimurium expressing the *Yersinia pestis* V antigen protects mice against plague. *Vaccine* 21, 3051-3057.
- Geissler, M., Gesien, A., Tokushige, K., and Wands, J.R. (1997). Enhancement of cellular and humoral immune responses to hepatitis C virus core protein using DNA-based vaccines augmented with cytokine-expressing plasmids. *J. Immunol.* 158, 1231-1237.
- Germanier, R. and Fuer, E. (1975). Isolation and characterization of Gal E mutant Ty 21a of *Salmonella typhi*: a candidate strain for a live, oral typhoid vaccine. *J. Infect. Dis.* 131, 553-558.
- Glenny, A.T., Buttle, G.A.H., and Stevens, M.F. (1931) Rate of disappearance of diphtheria toxoids injected into rabbits and guinea pigs: Toxoid precipitated with alum. *J. Path. Bact.* 34, 267-275.

- Grillot-Courvalin,C., Goussard,S., Huetz,F., Ojcius,D.M., and Courvalin,P. (1998). Functional gene transfer from intracellular bacteria to mammalian cells. *Nat. Biotechnol.* 16, 862-866.
- Grillot-Courvalin,C., Goussard,S., and Courvalin,P. (1999). Bacteria as gene delivery vectors for mammalian cells. *Curr. Opin. Biotechnol.* 10, 477-481.
- Grillot-Courvalin,C., Goussard,S., and Courvalin,P. (2002). Wild-type intracellular bacteria deliver DNA into mammalian cells. *Cell Microbiol.* 4, 177-186.
- Gupta,R.K. (1998). Aluminum compounds as vaccine adjuvants. *Adv. Drug Deliv. Rev.* 32, 155-172.
- Gurunathan,S., Klinman,D.M., and Seder,R.A. (2000). DNA vaccines: immunology, application, and optimization*. *Annu. Rev. Immunol.* 18, 927-974.
- Guzman,C.A., Rohde,M., Chakraborty,T., Domann,E., Hudel,M., Wehland,J., and Timmis,K.N. (1995). Interaction of *Listeria monocytogenes* with mouse dendritic cells. *Infect. Immun.* 63, 3665-3673.
- Haddad,D., Liljeqvist,S., Kumar,S., Hansson,M., Stahl,S., Perlmann,H., Perlmann,P., and Berzins,K. (1995). Surface display compared to periplasmic expression of a malarial antigen in *Salmonella typhimurium* and its implications for immunogenicity. *FEMS Immunol. Med. Microbiol.* 12, 175-186.
- Hajishengallis,G., Harokopakis,E., Hollingshead,S.K., Russell,M.W., and Michalek,S.M. (1996). Construction and oral immunogenicity of a *Salmonella typhimurium* strain expressing a streptococcal adhesin linked to the A2/B subunits of cholera toxin. *Vaccine* 14, 1545-1548.
- Haq,T.A., Mason,H.S., Clements,J.D., and Arntzen,C.J. (1995). Oral immunization with a recombinant bacterial antigen produced in transgenic plants. *Science* 268, 714-716.
- Hassan,J.O. and Curtiss,R., III (1996). Effect of vaccination of hens with an avirulent strain of *Salmonella typhimurium* on immunity of progeny challenged with wild-Type *Salmonella* strains. *Infect. Immun.* 64, 938-944.
- Heath,W.R. and Carbone,F.R. (2001). Cross-presentation, dendritic cells, tolerance and immunity. *Annu. Rev. Immunol.* 19, 47-64.
- Heinemann,J.A. and Sprague,G.F., Jr. (1989). Bacterial conjugative plasmids mobilize DNA transfer between bacteria and yeast. *Nature* 340, 205-209.
- Hense,M., Domann,E., Krusch,S., Wachholz,P., Dittmar,K.E., Rohde,M., Wehland,J., Chakraborty,T., and Weiss,S. (2001). Eukaryotic expression plasmid transfer from the intracellular bacterium *Listeria monocytogenes* to host cells. *Cell Microbiol.* 3, 599-609.
- Hersh,D., Monack,D.M., Smith,M.R., Ghoris,N., Falkow,S., and Zychlinsky,A. (1999). The *Salmonella* invasin SipB induces macrophage apoptosis by binding to caspase-1. *Proc. Natl. Acad. Sci. U. S A* 96, 2396-2401.

- Hess,J., Gentschev,I., Miko,D., Welzel,M., Ladel,C., Goebel,W., and Kaufmann,S.H. (1996). Superior efficacy of secreted over somatic antigen display in recombinant *Salmonella* vaccine induced protection against listeriosis. *Proc. Natl. Acad. Sci. U. S A* 93, 1458-1463.
- Hohlfeld,R., Engel,A.G., Li,K., and Harper,M.C. (1991). Polymyositis mediated by T lymphocytes that express the gamma/delta receptor. *N. Engl. J. Med.* 324, 877-881.
- Hohmann,A.W., Schmidt,G., and Rowley,D. (1978). Intestinal colonization and virulence of *Salmonella* in mice. *Infect. Immun.* 22, 763-770.
- Hohmann,E.L., Oletta,C.A., Loomis,W.P., and Miller,S.I. (1995). Macrophage-inducible expression of a model antigen in *Salmonella typhimurium* enhances immunogenicity. *Proc. Natl. Acad. Sci. U. S A* 92, 2904-2908.
- Hoiseth,S.K. and Stocker,B.A. (1981). Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature* 291, 238-239.
- Hone,D., Attridge,S., van den,B.L., and Hackett,J. (1988). A chromosomal integration system for stabilization of heterologous genes in *Salmonella* based vaccine strains. *Microb. Pathog.* 5, 407-418.
- Hopkins,S., Kraehenbuhl,J.P., Schodel,F., Potts,A., Peterson,D., de Grandi,P., and Nardelli-Haeffliger,D. (1995). A recombinant *Salmonella typhimurium* vaccine induces local immunity by four different routes of immunization. *Infect. Immun.* 63, 3279-3286.
- Hopkins,S.A., Niedergang,F., Corthesy-Theulaz,I.E., and Kraehenbuhl,J.P. (2000). A recombinant *Salmonella typhimurium* vaccine strain is taken up and survives within murine Peyer's patch dendritic cells. *Cell Microbiol.* 2, 59-68.
- Hormaeche,C.E. (1979). Natural resistance to *Salmonella typhimurium* in different inbred mouse strains. *Immunology* 37, 311-318.
- Hormaeche,C.E. (1991). Live attenuated *Salmonella* vaccines and their potential as oral combined vaccines carrying heterologous antigens. *J. Immunol. Methods* 142, 113-120.
- Hsu,S.C., Schadeck,E.B., Delmas,A., Shaw,M., and Steward,M.W. (1996). Linkage of a fusion peptide to a CTL epitope from the nucleoprotein of measles virus enables incorporation into ISCOMs and induction of CTL responses following intranasal immunization. *Vaccine* 14, 1159-1166.
- Huygen,K., Content,J., Denis,O., Montgomery,D.L., Yawman,A.M., Deck,R.R., DeWitt,C.M., Orme,I.M., Baldwin,S., D'Souza,C., Drowart,A., Lozes,E., Vandenbussche,P., Van Vooren,J.P., Liu,M.A., and Ulmer,J.B. (1996). Immunogenicity and protective efficacy of a tuberculosis DNA vaccine. *Nat. Med.* 2, 893-898.
- Ikonomidis,G., Portnoy,D.A., Gerhard,W., and Paterson,Y. (1997). Influenza-specific immunity induced by recombinant *Listeria monocytogenes* vaccines. *Vaccine* 15, 433-440.

- Iwasaki,A., Torres,C.A., Ohashi,P.S., Robinson,H.L., and Barber,B.H. (1997). The dominant role of bone marrow-derived cells in CTL induction following plasmid DNA immunization at different sites. *J. Immunol.* 159, 11-14.
- Jakob,T., Walker,P.S., Krieg,A.M., Udey,M.C., and Vogel,J.C. (1998). Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: a role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA. *J. Immunol.* 161, 3042-3049.
- Jenner, E. (1798) *An Inquiry into the Cause and Effects of the Variolae Vaccine*. Low, London.
- Jensen,E.R., Shen,H., Wettstein,F.O., Ahmed,R., and Miller,J.F. (1997). Recombinant *Listeria monocytogenes* as a live vaccine vehicle and a probe for studying cell-mediated immunity. *Immunol. Rev.* 158, 147-157.
- Jones,B.D., Paterson,H.F., Hall,A., and Falkow,S. (1993). *Salmonella typhimurium* induces membrane ruffling by a growth factor-receptor-independent mechanism. *Proc. Natl. Acad. Sci. U. S A* 90, 10390-10394.
- Jones,B.D., Ghori,N., and Falkow,S. (1994). *Salmonella typhimurium* initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. *J. Exp. Med.* 180, 15-23.
- Jones,P.W., Dougan,G., Hayward,C., Mackenzie,N., Collins,P., and Chatfield,S.N. (1991). Oral vaccination of calves against experimental salmonellosis using a double *aro* mutant of *Salmonella typhimurium*. *Vaccine* 9, 29-34.
- Kaech,S.M., Wherry,E.J., and Ahmed,R. (2002). Effector and memory T-cell differentiation: implications for vaccine development. *Nat. Rev. Immunol.* 2, 251-262.
- Katz,E. and Moss,B. (1997). Immunogenicity of recombinant vaccinia viruses that display the HIV type 1 envelope glycoprotein on the surface of infectious virions. *AIDS Res. Hum. Retroviruses* 13, 1497-1500.
- Katz,E., Wolffe,E.J., and Moss,B. (1997). The cytoplasmic and transmembrane domains of the vaccinia virus B5R protein target a chimeric human immunodeficiency virus type 1 glycoprotein to the outer envelope of nascent vaccinia virions. *J. Virol.* 71, 3178-3187.
- Khan,C.M., Villarreal-Ramos,B., Pierce,R.J., Riveau,G., Demarco,d.H., McNeill,H., Ali,T., Fairweather,N., Chatfield,S., Capron,A., and . (1994). Construction, expression, and immunogenicity of the *Schistosoma mansoni* P28 glutathione S-transferase as a genetic fusion to tetanus toxin fragment C in a live *Aro* attenuated vaccine strain of *Salmonella*. *Proc. Natl. Acad. Sci. U. S A* 91, 11261-11265.
- Khan,I.A. and Casciotti,L. (1999). IL-15 prolongs the duration of CD8+ T cell-mediated immunity in mice infected with a vaccine strain of *Toxoplasma gondii*. *J. Immunol.* 163, 4503-4509.

- Kim,D.W., Uetsuki,T., Kaziro,Y., Yamaguchi,N., and Sugano,S. (1990). Use of the human elongation factor 1 alpha promoter as a versatile and efficient expression system. *Gene* 91, 217-223.
- Kim,J.J., Ayyavoo,V., Bagarazzi,M.L., Chattergoon,M.A., Dang,K., Wang,B., Boyer,J.D., and Weiner,D.B. (1997). In vivo engineering of a cellular immune response by coadministration of IL-12 expression vector with a DNA immunogen. *J. Immunol.* 158, 816-826.
- Kleckner,N., Chan,R.K., Tye,B.K., and Botstein,D. (1975). Mutagenesis by insertion of a drug-resistance element carrying an inverted repetition. *J. Mol. Biol.* 97, 561-575.
- Kleckner,N. (1977). Translocatable elements in procaryotes. *Cell* 11, 11-23.
- Klee,S.R., Tzschaschel,B.D., Singh,M., Falt,I., Lindberg,A.A., Timmis,K.N., and Guzman,C.A. (1997). Construction and characterization of genetically-marked bivalent anti-Shigella dysenteriae 1 and anti-Shigella flexneri Y live vaccine candidates. *Microb. Pathog.* 22, 363-376.
- Klinman,D.M., Yi,A.K., Beaucage,S.L., Conover,J., and Krieg,A.M. (1996). CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc. Natl. Acad. Sci. U. S A* 93, 2879-2883.
- Klinman,D.M., Yamshchikov,G., and Ishigatsubo,Y. (1997). Contribution of CpG motifs to the immunogenicity of DNA vaccines. *J. Immunol.* 158, 3635-3639.
- Klinman,D.M., Barnhart,K.M., and Conover,J. (1999). CpG motifs as immune adjuvants. *Vaccine* 17, 19-25.
- Kohbata,S., Yokoyama,H., and Yabuuchi,E. (1986). Cytopathogenic effect of Salmonella typhi GIFU 10007 on M cells of murine ileal Peyer's patches in ligated ileal loops: an ultrastructural study. *Microbiol. Immunol.* 30, 1225-1237.
- Krieg,A.M., Yi,A.K., Matson,S., Waldschmidt,T.J., Bishop,G.A., Teasdale,R., Koretzky,G.A., and Klinman,D.M. (1995a). CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374, 546-549.
- Krieg,A.M., Yi,A.K., Matson,S., Waldschmidt,T.J., Bishop,G.A., Teasdale,R., Koretzky,G.A., and Klinman,D.M. (1995b). CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374, 546-549.
- Krieg,A.M., Love-Homan,L., Yi,A.K., and Harty,J.T. (1998). CpG DNA induces sustained IL-12 expression in vivo and resistance to Listeria monocytogenes challenge. *J. Immunol.* 161, 2428-2434.
- Krieg,A.M. (1999). Direct immunologic activities of CpG DNA and implications for gene therapy. *J. Gene Med.* 1, 56-63.
- Krusch,S., Domann,E., Frings,M., Zelmer,A., Diener,M., Chakraborty,T., and Weiss,S. (2002). Listeria monocytogenes mediated CFTR transgene transfer to mammalian cells. *J. Gene Med.* 4, 655-667.

- Kuhrober,A., Wild,J., Pudollek,H.P., Chisari,F.V., and Reimann,J. (1997). DNA vaccination with plasmids encoding the intracellular (HBcAg) or secreted (HBeAg) form of the core protein of hepatitis B virus primes T cell responses to two overlapping Kb- and Kd-restricted epitopes. *Int. Immunol.* 9, 1203-1212.
- Lang,H. and Korhonen,T.K. (1997). The OmpS maltoporin of *Vibrio cholerae* as carrier of foreign epitopes. *Behring Inst. Mitt.* 400-409.
- Leimeister-Wachter,M. and Chakraborty,T. (1989). Detection of listeriolysin, the thiol-dependent hemolysin in *Listeria monocytogenes*, *Listeria ivanovii*, and *Listeria seeligeri*. *Infect. Immun.* 57, 2350-2357.
- Lessl,M. and Lanka,E. (1994). Common mechanisms in bacterial conjugation and Ti-mediated T-DNA transfer to plant cells. *Cell* 77, 321-324.
- Letvin,N.L., Montefiori,D.C., Yasutomi,Y., Perry,H.C., Davies,M.E., Lekutis,C., Alroy,M., Freed,D.C., Lord,C.I., Handt,L.K., Liu,M.A., and Shiver,J.W. (1997). Potent, protective anti-HIV immune responses generated by bimodal HIV envelope DNA plus protein vaccination. *Proc. Natl. Acad. Sci. U. S A* 94, 9378-9383.
- Levine,M.M., Ferreccio,C., Black,R.E., and Germanier,R. (1987). Large-scale field trial of Ty21a live oral typhoid vaccine in enteric-coated capsule formulation. *Lancet* 1, 1049-1052.
- Levine,M.M., Galen,J., Barry,E., Noriega,F., Tacket,C., Sztein,M., Chatfield,S., Dougan,G., Losonsky,G., and Kotloff,K. (1997). Attenuated *Salmonella typhi* and *Shigella* as live oral vaccines and as live vectors. *Behring Inst. Mitt.* 120-123.
- Levine,M.M., Ferreccio,C., Abrego,P., Martin,O.S., Ortiz,E., and Cryz,S. (1999). Duration of efficacy of Ty21a, attenuated *Salmonella typhi* live oral vaccine. *Vaccine* 17 Suppl 2, S22-S27.
- Levine,M.M., Galen,E., Pasetti,M.F., Tacket,C.O., and Sztein,M.B. (2002). Attenuated *Salmonella* and *Shigella* Live Vectors. In *Vaccine Delivery Strategies*.
- Lewin B, ed. 1994. *Genes V*. Oxford Univ. Press/Cell. 5th ed.
- Liang,X., Hartikka,J., Sukhu,L., Manthorpe,M., and Hobart,P. (1996). Novel, high expressing and antibiotic-controlled plasmid vectors designed for use in gene therapy. *Gene Ther.* 3, 350-356.
- Liljeqvist,S. and Stahl,S. (1999). Production of recombinant subunit vaccines: protein immunogens, live delivery systems and nucleic acid vaccines. *J. Biotechnol.* 73, 1-33.
- Liu,M.A., Yasutomi,Y., Davies,M.E., Perry,H.C., Freed,D.C., Letvin,N.L., and Shiver,J.W. (1996). Vaccination of mice and nonhuman primates using HIV-gene-containing DNA. *Antibiot. Chemother.* 48, 100-104.

- Lode,H.N., Pertl,U., Xiang,R., Gaedicke,G., and Reisfeld,R.A. (2000). Tyrosine hydroxylase-based DNA-vaccination is effective against murine neuroblastoma. *Med. Pediatr. Oncol.* 35, 641-646.
- Londono,L.P., Chatfield,S., Tindle,R.W., Herd,K., Gao,X.M., Frazer,I., and Dougan,G. (1996). Immunisation of mice using *Salmonella typhimurium* expressing human papillomavirus type 16 E7 epitopes inserted into hepatitis B virus core antigen. *Vaccine* 14, 545-552.
- Lopez-Cabrera,M., Nueda,A., Vara,A., Garcia-Aguilar,J., Tugores,A., and Corbi,A.L. (1993). Characterization of the p150,95 leukocyte integrin alpha subunit (CD11c) gene promoter. Identification of cis-acting elements. *J. Biol. Chem.* 268, 1187-1193.
- Lubitz,W. (2001). Bacterial ghosts as carrier and targeting systems. *Expert. Opin. Biol. Ther.* 1, 765-771.
- Mackett,M., Smith,G.L., and Moss,B. (1982). Vaccinia virus: a selectable eukaryotic cloning and expression vector. *Proc. Natl. Acad. Sci. U. S A* 79, 7415-7419.
- Maeurer,M.J., Trinder,P., Hommel,G., Walter,W., Freitag,K., Atkins,D., and Storkel,S. (2000). Interleukin-7 or interleukin-15 enhances survival of *Mycobacterium tuberculosis*-infected mice. *Infect. Immun.* 68, 2962-2970.
- Makela, PH. (2000). Vaccines, coming of age after 200 years. *FEMS Microbiol Rev.* 2000 Jan;24(1):9-20
- Manickan,E., Rouse,R.J., Yu,Z., Wire,W.S., and Rouse,B.T. (1995). Genetic immunization against herpes simplex virus. Protection is mediated by CD4+ T lymphocytes. *J. Immunol.* 155, 259-265.
- Mannino,R.J., Canki,M., Feketeova,E., Scolpino,A.J., Wang,Z., Zhang,F., Kheiri,M.T., and Gould-Fogerite,S. (1998). Targeting immune response induction with cochleate and liposome-based vaccines. *Adv. Drug Deliv. Rev.* 32, 273-287.
- Manthorpe,M., Cornefert-Jensen,F., Hartikka,J., Felgner,J., Rundell,A., Margalith,M., and Dwarki,V. (1993). Gene therapy by intramuscular injection of plasmid DNA: studies on firefly luciferase gene expression in mice. *Hum. Gene Ther.* 4, 419-431.
- Marrack,P., Kappler,J., and Mitchell,T. (1999). Type I interferons keep activated T cells alive. *J. Exp. Med.* 189, 521-530.
- Martins,L.P., Lau,L.L., Asano,M.S., and Ahmed,R. (1995). DNA vaccination against persistent viral infection. *J. Virol.* 69, 2574-2582.
- Mastroeni,P., Vazquez-Torres,A., Fang,F.C., Xu,Y., Khan,S., Hormaeche,C.E., and Dougan,G. (2000). Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival in vivo. *J. Exp. Med.* 192, 237-248.

- Matzinger,P. (1991). The JAM test. A simple assay for DNA fragmentation and cell death. *J. Immunol. Methods* 145, 185-192.
- McMahon-Pratt,D., Rodriguez,D., Rodriguez,J.R., Zhang,Y., Manson,K., Bergman,C., Rivas,L., Rodriguez,J.F., Lohman,K.L., Ruddle,N.H., and . (1993). Recombinant vaccinia viruses expressing GP46/M-2 protect against *Leishmania* infection. *Infect. Immun.* 61, 3351-3359.
- McSorley,S.J., Xu,D., and Liew,F.Y. (1997). Vaccine efficacy of *Salmonella* strains expressing glycoprotein 63 with different promoters. *Infect. Immun.* 65, 171-178.
- Medina,E., Paglia,P., Nikolaus,T., Muller,A., Hensel,M., and Guzman,C.A. (1999). Pathogenicity island 2 mutants of *Salmonella typhimurium* are efficient carriers for heterologous antigens and enable modulation of immune responses. *Infect. Immun.* 67, 1093-1099.
- Medina,E., Paglia,P., Rohde,M., Colombo,M.P., and Guzman,C.A. (2000). Modulation of host immune responses stimulated by *Salmonella* vaccine carrier strains by using different promoters to drive the expression of the recombinant antigen. *Eur. J. Immunol.* 30, 768-777.
- Medina,E. and Guzman,C.A. (2001). Use of live bacterial vaccine vectors for antigen delivery: potential and limitations. *Vaccine* 19, 1573-1580.
- Medzhitov,R. and Janeway,C.A., Jr. (1998). Innate immune recognition and control of adaptive immune responses. *Semin. Immunol.* 10, 351-353.
- Messina,J.P., Gilkeson,G.S., and Pisetsky,D.S. (1991). Stimulation of in vitro murine lymphocyte proliferation by bacterial DNA. *J. Immunol.* 147, 1759-1764.
- Mielcarek,N., Riveau,G., Remoue,F., Antoine,R., Capron,A., and Locht,C. (1998). Homologous and heterologous protection after single intranasal administration of live attenuated recombinant *Bordetella pertussis*. *Nat. Biotechnol.* 16, 454-457.
- Miller,S.I., Kukral,A.M., and Mekalanos,J.J. (1989). A two-component regulatory system (phoP phoQ) controls *Salmonella typhimurium* virulence. *Proc. Natl. Acad. Sci. U. S A* 86, 5054-5058.
- Minor,P.D. (1992). The molecular biology of poliovaccines. *J. Gen. Virol.* 73 (Pt 12), 3065-3077.
- Mittal,S.K., Papp,Z., Tikoo,S.K., Baca-Estrada,M.E., Yoo,D., Benko,M., and Babiuk,L.A. (1996). Induction of systemic and mucosal immune responses in cotton rats immunized with human adenovirus type 5 recombinants expressing the full and truncated forms of bovine herpesvirus type 1 glycoprotein gD. *Virology* 222, 299-309.
- Monack,D.M., Raupach,B., Hromockyj,A.E., and Falkow,S. (1996). *Salmonella typhimurium* invasion induces apoptosis in infected macrophages. *Proc. Natl. Acad. Sci. U. S A* 93, 9833-9838.
- Monack,D.M., Hersh,D., Gori,N., Bouley,D., Zychlinsky,A., and Falkow,S. (2000). *Salmonella* exploits caspase-1 to colonize Peyer's patches in a murine typhoid model. *J. Exp. Med.* 192, 249-258.

- Monack,D.M., Detweiler,C.S., and Falkow,S. (2001). Salmonella pathogenicity island 2-dependent macrophage death is mediated in part by the host cysteine protease caspase-1. *Cell Microbiol.* 3, 825-837.
- Montgomery,D.L., Shiver,J.W., Leander,K.R., Perry,H.C., Friedman,A., Martinez,D., Ulmer,J.B., Donnelly,J.J., and Liu,M.A. (1993). Heterologous and homologous protection against influenza A by DNA vaccination: optimization of DNA vectors. *DNA Cell Biol.* 12, 777-783.
- Montosi,G., Paglia,P., Garuti,C., Guzman,C.A., Bastin,J.M., Colombo,M.P., and Pietrangelo,A. (2000). Wild-type HFE protein normalizes transferrin iron accumulation in macrophages from subjects with hereditary hemochromatosis. *Blood* 96, 1125-1129.
- Morein,F. and Bengtsson,K.L. (1998). Functional aspects of iscoms. *Immunol. Cell Biol.* 76, 295-299.
- Mowat,A.M., Smith,R.E., Donachie,A.M., Furrie,E., Grdic,D., and Lycke,N. (1999). Oral vaccination with immune stimulating complexes. *Immunol. Lett.* 65, 133-140.
- Mukkur,T.K., McDowell,G.H., Stocker,B.A., and Lascelles,A.K. (1987). Protection against experimental salmonellosis in mice and sheep by immunisation with aromatic-dependent Salmonella typhimurium. *J. Med. Microbiol.* 24, 11-19.
- Mullis,K., Faloona,F., Scharf,S., Saiki,R., Horn,G., and Erlich,H. (1986). Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harb. Symp. Quant. Biol.* 51 Pt 1, 263-273.
- Nakayama,N., Kaziro,Y., Arai,K., and Matsumoto,K. (1988). Role of STE genes in the mating factor signaling pathway mediated by GPA1 in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 8, 3777-3783.
- Nenoi,M., Mita,K., Ichimura,S., Cartwright,I.L., Takahashi,E., Yamauchi,M., and Tsuji,H. (1996). Heterogeneous structure of the polyubiquitin gene UbC of HeLa S3 cells. *Gene* 175, 179-185.
- Neutra,M.R. and Kraehenbuhl,J.P. (1992). M cell-mediated antigen transport and monoclonal IgA antibodies for mucosal immune protection. *Adv. Exp. Med. Biol.* 327, 143-150.
- Neutra,M.R. and Kraehenbuhl,J.P. (1994). Mucosal immunization via M cells for production of protective secretory IgA antibodies. *Am. J. Trop. Med. Hyg.* 50, 10-13.
- Neutra,M.R., Frey,A., and Kraehenbuhl,J.P. (1996a). Epithelial M cells: gateways for mucosal infection and immunization. *Cell* 86, 345-348.
- Neutra,M.R., Pringault,E., and Kraehenbuhl,J.P. (1996b). Antigen sampling across epithelial barriers and induction of mucosal immune responses. *Annu. Rev. Immunol.* 14, 275-300.
- Newton,S.M., Jacob,C.O., and Stocker,B.A. (1989). Immune response to cholera toxin epitope inserted in Salmonella flagellin. *Science* 244, 70-72.

- Niethammer,A.G., Xiang,R., Ruehlmann,J.M., Lode,H.N., Dolman,C.S., Gillies,S.D., and Reisfeld,R.A. (2001). Targeted interleukin 2 therapy enhances protective immunity induced by an autologous oral DNA vaccine against murine melanoma. *Cancer Res.* 61, 6178-6184.
- Noriega,F.R., Wang,J.Y., Losonsky,G., Maneval,D.R., Hone,D.M., and Levine,M.M. (1994). Construction and characterization of attenuated delta aroA delta virG *Shigella flexneri* 2a strain CVD 1203, a prototype live oral vaccine. *Infect. Immun.* 62, 5168-5172.
- Noriega,F.R., Losonsky,G., Lauderbaugh,C., Liao,F.M., Wang,J.Y., and Levine,M.M. (1996). Engineered deltaguaB-A deltavirG *Shigella flexneri* 2a strain CVD 1205: construction, safety, immunogenicity, and potential efficacy as a mucosal vaccine. *Infect. Immun.* 64, 3055-3061.
- Norman,J.A., Hobart,P., Manthorpe,M., Felgner,P., and Wheeler,C. (1997). Development of improved vectors for DNA-based immunization and other gene therapy applications. *Vaccine* 15, 801-803.
- Ohl,M.E. and Miller,S.I. (2001). *Salmonella*: a model for bacterial pathogenesis. *Annu. Rev. Med.* 52, 259-274.
- Paglia,P., Arioli,I., Frahm,N., Chakraborty,T., Colombo,M.P., and Guzman,C.A. (1997). The defined attenuated *Listeria monocytogenes* delta mp12 mutant is an effective oral vaccine carrier to trigger a long-lasting immune response against a mouse fibrosarcoma. *Eur. J. Immunol.* 27, 1570-1575.
- Paglia,P., Medina,E., Arioli,I., Guzman,C.A., and Colombo,M.P. (1998). Gene transfer in dendritic cells, induced by oral DNA vaccination with *Salmonella typhimurium*, results in protective immunity against a murine fibrosarcoma. *Blood* 92, 3172-3176.
- Paglia,P., Terrazzini,N., Schulze,K., Guzman,C.A., and Colombo,M.P. (2000). In vivo correction of genetic defects of monocyte/macrophages using attenuated *Salmonella* as oral vectors for targeted gene delivery. *Gene Ther.* 7, 1725-1730.
- Pamer,E.G., Harty,J.T., and Bevan,M.J. (1991). Precise prediction of a dominant class I MHC-restricted epitope of *Listeria monocytogenes*. *Nature* 353, 852-855.
- Pan,Z.K., Ikonomidis,G., Lazenby,A., Pardoll,D., and Paterson,Y. (1995). A recombinant *Listeria monocytogenes* vaccine expressing a model tumour antigen protects mice against lethal tumour cell challenge and causes regression of established tumours. *Nat. Med.* 1, 471-477.
- Pasetti,M.F., Anderson,R.J., Noriega,F.R., Levine,M.M., and Sztein,M.B. (1999). Attenuated deltaguaBA *Salmonella typhi* vaccine strain CVD 915 as a live vector utilizing prokaryotic or eukaryotic expression systems to deliver foreign antigens and elicit immune responses. *Clin. Immunol.* 92, 76-89.
- Pasteur, L. (1880) De l'attenuation du virus du cholera des poules. *C.R. Acad. Sci. Paris* 91, 673-680.
- Pasteur, L. (1885) Methode pour prevenir la rage apres morsure. *C.R. Acad. Sci. Paris* 101, 765-772.

- Peltola,H., Kilpi,T., and Anttila,M. (1992). Rapid disappearance of Haemophilus influenzae type b meningitis after routine childhood immunisation with conjugate vaccines. *Lancet* 340, 592-594.
- Pfarr,D.S., Rieser,L.A., Woychik,R.P., Rottman,F.M., Rosenberg,M., and Reff,M.E. (1986). Differential effects of polyadenylation regions on gene expression in mammalian cells. *DNA* 5, 115-122.
- Pilgrim,S., Stritzker,J., Schoen,C., Kolb-Maurer,A., Geginat,G., Loessner,M.J., Gentschev,I., and Goebel,W. (2003). Bactofection of mammalian cells by *Listeria monocytogenes*: improvement and mechanism of DNA delivery. *Gene Ther.* 10, 2036-2045.
- Pittard, A.J. 1990. Biosynthesis of the aromatic acids. In *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular biology. Ed. by F.C. Neidhardt, Washington, D.C.: American Society for Microbiology.
- Pizza,G., de Vinci,C., and Viza,D. (2002). Immunotherapeutic approaches for renal cancer. *Folia Biol. (Praha)* 48, 167-181.
- Porgador,A., Irvine,K.R., Iwasaki,A., Barber,B.H., Restifo,N.P., and Germain,R.N. (1998). Predominant role for directly transfected dendritic cells in antigen presentation to CD8+ T cells after gene gun immunization. *J. Exp. Med.* 188, 1075-1082.
- Powell, R.J., Lewis, G.K., Hone, D.M.: Introduction of eukaryotic expression cassettes into animal cells using bacterial vector delivery system. In: *Vaccines 96: Molecular approaches to the control of infectious disease*. Cold Spring Harbor Laboratory Press, Cold Spring Habor, NY, pp 183-187 (1996).
- Prince,A.M. (1996). Prevention of liver cancer and cirrhosis by vaccines. *Clin. Lab Med.* 16, 493-505.
- Qin,L., Ding,Y., Pahud,D.R., Chang,E., Imperiale,M.J., and Bromberg,J.S. (1997). Promoter attenuation in gene therapy: interferon-gamma and tumor necrosis factor-alpha inhibit transgene expression. *Hum. Gene Ther.* 8, 2019-2029.
- Radford,K.J., Higgins,D.E., Pasquini,S., Cheadle,E.J., Carta,L., Jackson,A.M., Lemoine,N.R., and Vassaux,G. (2002). A recombinant *E. coli* vaccine to promote MHC class I-dependent antigen presentation: application to cancer immunotherapy. *Gene Ther.* 9, 1455-1463.
- Radford,K.J., Jackson,A.M., Wang,J.H., Vassaux,G., and Lemoine,N.R. (2003). Recombinant *E. coli* efficiently delivers antigen and maturation signals to human dendritic cells: presentation of MART1 to CD8+ T cells. *Int. J. Cancer* 105, 811-819.
- Ramon,G. (1924). Sur la toxine et sur Lánatoxine diphtheriques. *Ann. Inst. Pasteur.* 38, 1-8.
- Redman,T.K., Harmon,C.C., and Michalek,S.M. (1996). Oral immunization with recombinant *Salmonella typhimurium* expressing surface protein antigen A (SpaA) of *Streptococcus sobrinus*: effects of the *Salmonella* virulence plasmid on the induction of protective and sustained humoral responses in rats. *Vaccine* 14, 868-878.

- Rescigno,M., Urbano,M., Valzasina,B., Francolini,M., Rotta,G., Bonasio,R., Granucci,F., Kraehenbuhl,J.P., and Ricciardi-Castagnoli,P. (2001). Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat. Immunol.* 2, 361-367.
- Rettinger,S.D., Kennedy,S.C., Wu,X., Saylor,R.L., Hafenrichter,D.G., Flye,M.W., and Ponder,K.P. (1994). Liver-directed gene therapy: quantitative evaluation of promoter elements by using in vivo retroviral transduction. *Proc. Natl. Acad. Sci. U. S A* 91, 1460-1464.
- Roberts,J.A. and Sockett,P.N. (1994). The socio-economic impact of human *Salmonella enteritidis* infection. *Int. J. Food Microbiol.* 21, 117-129.
- Robinson,H.L., Montefiori,D.C., Johnson,R.P., Manson,K.H., Kalish,M.L., Lifson,J.D., Rizvi,T.A., Lu,S., Hu,S.L., Mazzara,G.P., Panicali,D.L., Herndon,J.G., Glickman,R., Candido M.A., Lydy,S.L., Wyand,M.S., McClure,H.M. (1999). Neutralizing antibody-independent containment of immunodeficiency virus challenges by DNA priming and recombinant pox virus booster immunizations. *Nat. Med.* 5, 526-34.
- Robson,H.G. and Vas,S.I. (1972). Resistance of inbred mice to *Salmonella typhimurium*. *J. Infect. Dis.* 126, 378-386.
- Roman,M., Martin-Orozco,E., Goodman,J.S., Nguyen,M.D., Sato,Y., Ronaghy,A., Kornbluth,R.S., Richman,D.D., Carson,D.A., and Raz,E. (1997). Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat. Med.* 3, 849-854.
- Rosenkranz,C.D., Chiara,D., Agorio,C., Baz,A., Pasetti,M.F., Schreiber,F., Dematteis,S., Martinez,M., Sztein,M.B., and Chabalgoity,J.A. (2003). Towards new immunotherapies: targeting recombinant cytokines to the immune system using live attenuated *Salmonella*. *Vaccine* 21, 798-801.
- Rosenstein,N.E. and Perkins,B.A. (2000). Update on *Haemophilus influenzae* serotype b and meningococcal vaccines. *Pediatr. Clin. North Am.* 47, 337-52, vi.
- Ryd,M., Verma,N., and Lindberg,A.A. (1992). Induction of a humoral immune response to a Shiga toxin B subunit epitope expressed as a chimeric LamB protein in a *Shigella flexneri* live vaccine strain. *Microb. Pathog.* 12, 399-407.
- Sadoff,J.C., Ballou,W.R., Baron,L.S., Majarian,W.R., Brey,R.N., Hockmeyer,W.T., Young,J.F., Cryz,S.J., Ou,J., Lowell,G.H., and . (1988). Oral *Salmonella typhimurium* vaccine expressing circumsporozoite protein protects against malaria. *Science* 240, 336-338.
- Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, Second Edition (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).
- Sanger,F., Nicklen,S., and Coulson,A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U. S A* 74, 5463-5467.

- Sato,Y., Roman,M., Tighe,H., Lee,D., Corr,M., Nguyen,M.D., Silverman,G.J., Lotz,M., Carson,D.A., and Raz,E. (1996). Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* 273, 352-354.
- Schaffner,W. (1980). Direct transfer of cloned genes from bacteria to mammalian cells. *Proc. Natl. Acad. Sci. U. S A* 77, 2163-2167.
- Scharfmann,R., Atouf,F., Tazi,A., and Czernichow,P. (1994). Growth hormone and prolactin regulate the expression of nerve growth factor receptors in INS-1 cells. *Endocrinology* 134, 2321-2328.
- Schirmbeck,R., Bohm,W., Ando,K., Chisari,F.V., and Reimann,J. (1995). Nucleic acid vaccination primes hepatitis B virus surface antigen-specific cytotoxic T lymphocytes in nonresponder mice. *J. Virol.* 69, 5929-5934.
- Schlegel,H.G. (1992). *Allgemeine Mikrobiologie*. 7. Auflage (Georg Thieme Verlag Stuttgart, New York).
- Schodel,F., Will,H., Johansson,S., Sanchez,J., and Holmgren,J. (1991). Synthesis in *Vibrio cholerae* and secretion of hepatitis B virus antigens fused to *Escherichia coli* heat-labile enterotoxin subunit B. *Gene* 99, 255-259.
- Schodel,F., Kelly,S.M., Peterson,D.L., Milich,D.R., and Curtiss,R., III (1994a). Hybrid hepatitis B virus core-pre-S proteins synthesized in avirulent *Salmonella typhimurium* and *Salmonella typhi* for oral vaccination. *Infect. Immun.* 62, 1669-1676.
- Schodel,F., Wirtz,R., Peterson,D., Hughes,J., Warren,R., Sadoff,J., and Milich,D. (1994b). Immunity to malaria elicited by hybrid hepatitis B virus core particles carrying circumsporozoite protein epitopes. *J. Exp. Med.* 180, 1037-1046.
- Scott,P. and Trinchieri,G. (1997). IL-12 as an adjuvant for cell-mediated immunity. *Semin. Immunol.* 9, 285-291.
- Sedegah,M., Hedstrom,R., Hobart,P., and Hoffman,S.L. (1994). Protection against malaria by immunization with plasmid DNA encoding circumsporozoite protein. *Proc. Natl. Acad. Sci. U. S A* 91, 9866-9870.
- Sedegah,M., Jones,T.R., Kaur,M., Hedstrom,R., Hobart,P., Tine,J.A., and Hoffman,S.L. (1998). Boosting with recombinant vaccinia increases immunogenicity and protective efficacy of malaria DNA vaccine. *Proc. Natl. Acad. Sci. U. S A* 95, 7648-7653.
- Shata,M.T. and Hone,D.M. (2001). Vaccination with a *Shigella* DNA vaccine vector induces antigen-specific CD8(+) T cells and antiviral protective immunity. *J. Virol.* 75, 9665-9670.

- Shiau,A.L., Chen,Y.L., Liao,C.Y., Huang,Y.S., and Wu,C.L. (2001). Prothymosin alpha enhances protective immune responses induced by oral DNA vaccination against pseudorabies delivered by *Salmonella choleraesuis*. *Vaccine* 19, 3947-3956.
- Shiver,J.W., Perry,H.C., Davies,M.E., Freed,D.C., and Liu,M.A. (1995). Cytotoxic T lymphocyte and helper T cell responses following HIV polynucleotide vaccination. *Ann. N. Y. Acad. Sci.* 772, 198-208.
- Shiver,J.W., Davies,M.E., Yasutomi,Y., Perry,H.C., Freed,D.C., Letvin,N.L., and Liu,M.A. (1997). Anti-HIV env immunities elicited by nucleic acid vaccines. *Vaccine* 15, 884-887.
- Siebers,A. and Finlay,B.B. (1996). M cells and the pathogenesis of mucosal and systemic infections. *Trends Microbiol.* 4, 22-29.
- Silverstein,A.M. (1998). The history of Immunology. In Paul: *Fundamental Immunology*, fourth edition (Lippincott-Raven Publisher, Philadelphia, New York).
- Simard,C., Nadon,F., Seguin,C., Thien,N.N., Binz,H., Basso,J., Laliberte,J.F., and Trudel,M. (1997). Subgroup specific protection of mice from respiratory syncytial virus infection with peptides encompassing the amino acid region 174-187 from the G glycoprotein: the role of cysteinyl residues in protection. *Vaccine* 15, 423-432.
- Sims,S.H., Cha,Y., Romine,M.F., Gao,P.Q., Gottlieb,K., and Deisseroth,A.B. (1993). A novel interferon-inducible domain: structural and functional analysis of the human interferon regulatory factor 1 gene promoter. *Mol. Cell Biol.* 13, 690-702.
- Sin,J.I., Kim,J.J., Zhang,D., and Weiner,D.B. (2001). Modulation of cellular responses by plasmid CD40L: CD40L plasmid vectors enhance antigen-specific helper T cell type 1 CD4+ T cell-mediated protective immunity against herpes simplex virus type 2 in vivo. *Hum. Gene Ther.* 12, 1091-1102.
- Sizemore,D.R., Branstrom,A.A., and Sadoff,J.C. (1995). Attenuated *Shigella* as a DNA delivery vehicle for DNA-mediated immunization. *Science* 270, 299-302.
- Sizemore,D.R., Branstrom,A.A., and Sadoff,J.C. (1997). Attenuated bacteria as a DNA delivery vehicle for DNA-mediated immunization. *Vaccine* 15, 804-807.
- Slifka,M.K., Shen,H., Matloubian,M., Jensen,E.R., Miller,J.F., and Ahmed,R. (1996). Antiviral cytotoxic T-cell memory by vaccination with recombinant *Listeria monocytogenes*. *J. Virol.* 70, 2902-2910.
- Smith,B.P., Reina-Guerra,M., Hoiseth,S.K., Stocker,B.A., Habasha,F., Johnson,E., and Merritt,F. (1984). Aromatic-dependent *Salmonella typhimurium* as modified live vaccines for calves. *Am. J. Vet. Res.* 45, 59-66.
- Smith,R.E., Donachie,A.M., and Mowat,A.M. (1998). Immune stimulating complexes as mucosal vaccines. *Immunol. Cell Biol.* 76, 263-269.

- Sory,M.P. and Cornelis,G.R. (1990). Delivery of the cholera toxin B subunit by using a recombinant *Yersinia enterocolitica* strain as a live oral carrier. *Res. Microbiol.* 141, 921-929.
- Sprent,J., Zhang,X., Sun,S., and Tough,D. (2000). T-cell proliferation in vivo and the role of cytokines. *Philos. Trans. R. Soc. Lond B Biol. Sci.* 355, 317-322.
- Shrivastava,I.K. and Liu,M.A. (2003). Gene vaccines. *Ann. Intern. Med.* 138, 550-559.
- Stacey,K.J., Sweet,M.J., and Hume,D.A. (1996). Macrophages ingest and are activated by bacterial DNA. *J. Immunol.* 157, 2116-2122.
- Steinbach,G., Koch,H., Meyer,H., and Klaus,C. (1996). Influence of prior infection on the dynamics of bacterial counts in calves experimentally infected with *Salmonella dublin*. *Vet. Microbiol.* 48, 199-206.
- Stocker,B.A. (1988). Auxotrophic *Salmonella typhi* as live vaccine. *Vaccine* 6, 141-145.
- Strugnell,R.A., Maskell,D., Fairweather,N., Pickard,D., Cockayne,A., Penn,C., and Dougan,G. (1990). Stable expression of foreign antigens from the chromosome of *Salmonella typhimurium* vaccine strains. *Gene* 88, 57-63.
- Sutcliffe,J.G. (1979). Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. *Cold Spring Harb. Symp. Quant. Biol.* 43 Pt 1, 77-90.
- Tacket,C.O., Forrest,B., Morona,R., Attridge,S.R., LaBrooy,J., Tall,B.D., Reymann,M., Rowley,D., and Levine,M.M. (1990). Safety, immunogenicity, and efficacy against cholera challenge in humans of a typhoid-cholera hybrid vaccine derived from *Salmonella typhi* Ty21a. *Infect. Immun.* 58, 1620-1627.
- Tacket,C.O., Mason,H.S., Losonsky,G., Clements,J.D., Levine,M.M., and Arntzen,C.J. (1998). Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato. *Nat. Med.* 4, 607-609.
- Tacket,C.O., Galen,J., Sztein,M.B., Losonsky,G., Wyant,T.L., Nataro,J., Wasserman,S.S., Edelman,R., Chatfield,S., Dougan,G., and Levine,M.M. (2000). Safety and immune responses to attenuated *Salmonella enterica* serovar typhi oral live vector vaccines expressing tetanus toxin fragment C. *Clin. Immunol.* 97, 146-153.
- Takeuchi,A. (1967). Electron microscope studies of experimental *Salmonella* infection. I. Penetration into the intestinal epithelium by *Salmonella typhimurium*. *Am. J. Pathol.* 50, 109-136.
- Tan,J.T., Dudl,E., LeRoy,E., Murray,R., Sprent,J., Weinberg,K.I., and Surh,C.D. (2001). IL-7 is critical for homeostatic proliferation and survival of naive T cells. *Proc. Natl. Acad. Sci. U. S A* 98, 8732-8737.
- Tang,D.C., DeVit,M., and Johnston,S.A. (1992). Genetic immunization is a simple method for eliciting an immune response. *Nature* 356, 152-154.

- Tascon,R.E., Colston,M.J., Ragno,S., Stavropoulos,E., Gregory,D., and Lowrie,D.B. (1996). Vaccination against tuberculosis by DNA injection. *Nat. Med.* 2, 888-892.
- Thomson,S.A., Burrows,S.R., Misko,I.S., Moss,D.J., Coupar,B.E., and Khanna,R. (1998). Targeting a polypeptide protein incorporating multiple class II-restricted viral epitopes to the secretory/endocytic pathway facilitates immune recognition by CD4+ cytotoxic T lymphocytes: a novel approach to vaccine design. *J. Virol.* 72, 2246-2252.
- Torres,C.A., Iwasaki,A., Barber,B.H., and Robinson,H.L. (1997). Differential dependence on target site tissue for gene gun and intramuscular DNA immunizations. *J. Immunol.* 158, 4529-4532.
- Trieu-Cuot,P., Derlot,E., and Courvalin,P. (1993). Enhanced conjugative transfer of plasmid DNA from *Escherichia coli* to *Staphylococcus aureus* and *Listeria monocytogenes*. *FEMS Microbiol. Lett.* 109, 19-23.
- Tubulekas,I., Berglund,P., Fleeton,M., and Liljestrom,P. (1997). Alphavirus expression vectors and their use as recombinant vaccines: a minireview. *Gene* 190, 191-195.
- Uchijima,M., Yoshida,A., Nagata,T., and Koide,Y. (1998). Optimization of codon usage of plasmid DNA vaccine is required for the effective MHC class I-restricted T cell responses against an intracellular bacterium. *J. Immunol.* 161, 5594-5599.
- Uetsuki,T., Naito,A., Nagata,S., and Kaziro,Y. (1989). Isolation and characterization of the human chromosomal gene for polypeptide chain elongation factor-1 alpha. *J. Biol. Chem.* 264, 5791-5798.
- Ulmer,J.B., Donnelly,J.J., Parker,S.E., Rhodes,G.H., Felgner,P.L., Dwarki,V.J., Gromkowski,S.H., Deck,R.R., DeWitt,C.M., Friedman,A., and . (1993). Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* 259, 1745-1749.
- Ulmer,J.B., Deck,R.R., DeWitt,C.M., Friedman,A., Donnelly,J.J., and Liu,M.A. (1994). Protective immunity by intramuscular injection of low doses of influenza virus DNA vaccines. *Vaccine* 12, 1541-1544.
- Ulmer,J.B., Donnelly,J.J., and Liu,M.A. (1996). Toward the development of DNA vaccines. *Curr. Opin. Biotechnol.* 7, 653-658.
- Urashima,M., Suzuki,H., Yuza,Y., Akiyama,M., Ohno,N., and Eto,Y. (2000). An oral CD40 ligand gene therapy against lymphoma using attenuated *Salmonella typhimurium*. *Blood* 95, 1258-1263.
- Valenzuela,P., Medina,A., Rutter,W.J., Ammerer,G., and Hall,B.D. (1982). Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. *Nature* 298, 347-350.
- VanCott,J.L., Chatfield,S.N., Roberts,M., Hone,D.M., Hohmann,E.L., Pascual,D.W., Yamamoto,M., Kiyono,H., and McGhee,J.R. (1998). Regulation of host immune responses by modification of *Salmonella* virulence genes. *Nat. Med.* 4, 1247-1252.

- Vella, A.T., Dow, S., Potter, T.A., Kappler, J., and Marrack, P. (1998). Cytokine-induced survival of activated T cells in vitro and in vivo. *Proc. Natl. Acad. Sci. U. S. A.* 95, 3810-3815.
- Vinner, L., Nielsen, H.V., Bryder, K., Corbet, S., Nielsen, C., and Fomsgaard, A. (1999). Gene gun DNA vaccination with Rev-independent synthetic HIV-1 gp160 envelope gene using mammalian codons. *Vaccine* 17, 2166-2175.
- Viret, J.F., Cryz, S.J., Jr., and Favre, D. (1996). Expression of *Shigella sonnei* lipopolysaccharide in *Vibrio cholerae*. *Mol. Microbiol.* 19, 949-963.
- Wahdan, M.H., Serie, C., Cerisier, Y., Sallam, S., and Germanier, R. (1982). A controlled field trial of live *Salmonella typhi* strain Ty 21a oral vaccine against typhoid: three-year results. *J. Infect. Dis.* 145, 292-295.
- Wang, R.F. and Kushner, S.R. (1991). Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene* 100, 195-199.
- Wang, R., Doolan, D.L., Le, T.P., Hedstrom, R.C., Coonan, K.M., Charoenvit, Y., Jones, T.R., Hobart, P., Margalith, M., Ng, J., Weiss, W.R., Sedegah, M., de Taisne, C., Norman, J.A., Hoffman, S.L. (1998). Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. *Science* 16, 476-480.
- Wang, J.Y., Pasetti, M.F., Noriega, F.R., Anderson, R.J., Wasserman, S.S., Galen, J.E., Sztein, M.B., and Levine, M.M. (2001). Construction, genotypic and phenotypic characterization, and immunogenicity of attenuated DeltaguaBA *Salmonella enterica* serovar Typhi strain CVD 915. *Infect. Immun.* 69, 4734-4741.
- Wedemeyer, H., Gagneten, S., Davis, A., Bartenschlager, R., Feinstone, S., and Rehermann, B. (2001). Oral immunization with HCV-NS3-transformed *Salmonella*: induction of HCV-specific CTL in a transgenic mouse model. *Gastroenterology* 121, 1158-1166.
- Weiskirch, L.M. and Paterson, Y. (1997). *Listeria monocytogenes*: a potent vaccine vector for neoplastic and infectious disease. *Immunol. Rev.* 158, 159-169.
- Weiss, S. and Chakraborty, T. (2001). Transfer of eukaryotic expression plasmids to mammalian host cells by bacterial carriers. *Curr. Opin. Biotechnol.* 12, 467-472.
- Weiss, S. and Krusch, S. (2001). Bacteria-mediated transfer of eukaryotic expression plasmids into mammalian host cells. *Biol. Chem.* 382, 533-541.
- Weiss, S. (2003). Transfer of eukaryotic expression plasmids to mammalian hosts by attenuated *Salmonella* spp. *Int. J. Med. Microbiol.* 293, 95-106.

- Weth,R., Christ,O., Stevanovic,S., and Zoller,M. (2001). Gene delivery by attenuated *Salmonella typhimurium*: comparing the efficacy of helper versus cytotoxic T cell priming in tumor vaccination. *Cancer Gene Ther.* 8, 599-611.
- Whitmire,J.K. and Ahmed,R. (2000). Costimulation in antiviral immunity: differential requirements for CD4(+) and CD8(+) T cell responses. *Curr. Opin. Immunol.* 12, 448-455.
- Wild,J., Gruner,B., Metzger,K., Kuhrober,A., Pudollek,H.P., Hauser,H., Schirmbeck,R., and Reimann,J. (1998). Polyvalent vaccination against hepatitis B surface and core antigen using a dicistronic expression plasmid. *Vaccine* 16, 353-360.
- Williams,R.S., Johnston,S.A., Riedy,M., DeVit,M.J., McElligott,S.G., and Sanford,J.C. (1991). Introduction of foreign genes into tissues of living mice by DNA-coated microprojectiles. *Proc. Natl. Acad. Sci. U. S A* 88, 2726-2730.
- Wolff,J.A., Malone,R.W., Williams,P., Chong,W., Acsadi,G., Jani,A., and Felgner,P.L. (1990). Direct gene transfer into mouse muscle in vivo. *Science* 247, 1465-1468.
- Wong,L.P., Woo,P.C., Wu,A.Y., and Yuen,K.Y. (2002). DNA immunization using a secreted cell wall antigen Mp1p is protective against *Penicillium marneffei* infection. *Vaccine* 20, 2878-2886.
- Woo,P.C., Tsoi,H.W., Leung,H.C., Wong,L.P., Wong,S.S., Chan,E., and Yuen,K.Y. (2000). Enhancement by ampicillin of antibody responses induced by a protein antigen and a DNA vaccine carried by live-attenuated *Salmonella enterica* serovar Typhi. *Clin. Diagn. Lab Immunol.* 7, 596-599.
- Woo,P.C., Wong,L.P., Zheng,B.J., and Yuen,K.Y. (2001). Unique immunogenicity of hepatitis B virus DNA vaccine presented by live-attenuated *Salmonella typhimurium*. *Vaccine* 19, 2945-2954.
- Wu,Y. and Kipps,T.J. (1997). Deoxyribonucleic acid vaccines encoding antigens with rapid proteasome-dependent degradation are highly efficient inducers of cytolytic T lymphocytes. *J. Immunol.* 159, 6037-6043.
- Xiang,R., Lode,H.N., Chao,T.H., Ruehlmann,J.M., Dolman,C.S., Rodriguez,F., Whitton,J.L., Overwijk,W.W., Restifo,N.P., and Reisfeld,R.A. (2000). An autologous oral DNA vaccine protects against murine melanoma. *Proc. Natl. Acad. Sci. U. S A* 97, 5492-5497.
- Xiang,R., Primus,F.J., Ruehlmann,J.M., Niethammer,A.G., Silletti,S., Lode,H.N., Dolman,C.S., Gillies,S.D., and Reisfeld,R.A. (2001). A dual-function DNA vaccine encoding carcinoembryonic antigen and CD40 ligand trimer induces T cell-mediated protective immunity against colon cancer in carcinoembryonic antigen-transgenic mice. *J. Immunol.* 167, 4560-4565.
- Xiang,Z. and Ertl,H.C. (1995). Manipulation of the immune response to a plasmid-encoded viral antigen by coinoculation with plasmids expressing cytokines. *Immunity* 2, 129-135.

- Xiang,Z.Q., Spitalnik,S., Tran,M., Wunner,W.H., Cheng,J., and Ertl,H.C. (1994). Vaccination with a plasmid vector carrying the rabies virus glycoprotein gene induces protective immunity against rabies virus. *Virology* 199, 132-140.
- Xiang,Z.Q., Yang,Y., Wilson,J.M., and Ertl,H.C. (1996). A replication-defective human adenovirus recombinant serves as a highly efficacious vaccine carrier. *Virology* 219, 220-227.
- Xu,D. and Liew,F.Y. (1995). Protection against leishmaniasis by injection of DNA encoding a major surface glycoprotein, gp63, of *L. major*. *Immunology* 84, 173-176.
- Yajima,T., Nishimura,H., Ishimitsu,R., Watase,T., Busch,D.H., Pamer,E.G., Kuwano,H., and Yoshikai,Y. (2002). Overexpression of IL-15 in vivo increases antigen-driven memory CD8+ T cells following a microbe exposure. *J. Immunol.* 168, 1198-1203.
- Yamamoto,S., Yamamoto,T., Shimada,S., Kuramoto,E., Yano,O., Kataoka,T., and Tokunaga,T. (1992). DNA from bacteria, but not from vertebrates, induces interferons, activates natural killer cells and inhibits tumor growth. *Microbiol. Immunol.* 36, 983-997.
- Yancey,R.J., Breeding,S.A., and Lankford,C.E. (1979). Enterochelin (enterobactin): virulence factor for *Salmonella typhimurium*. *Infect. Immun.* 24, 174-180.
- Yokoyama,M., Zhang,J., and Whitton,J.L. (1995). DNA immunization confers protection against lethal lymphocytic choriomeningitis virus infection. *J. Virol.* 69, 2684-2688.
- Yuhua,L., Kunyuan,G., Hui,C., Yongmei,X., Chaoyang,S., Xun,T., and Daming,R. (2001). Oral cytokine gene therapy against murine tumor using attenuated *Salmonella typhimurium*. *Int. J. Cancer* 94, 438-443.
- Zhang,X., Sun,S., Hwang,I., Tough,D.F., and Sprent,J. (1998). Potent and selective stimulation of memory-phenotype CD8+ T cells in vivo by IL-15. *Immunity* 8, 591-599.
- Zheng,B., Woo,P.C., Ng,M., Tsoi,H., Wong,L., and Yuen,K. (2001). A crucial role of macrophages in the immune responses to oral DNA vaccination against hepatitis B virus in a murine model. *Vaccine* 20, 140-147.
- Zhu,N., Liggitt,D., Liu,Y., and Debs,R. (1993). Systemic gene expression after intravenous DNA delivery into adult mice. *Science* 261, 209-211.
- Zoller,M. and Christ,O. (2001). Prophylactic tumor vaccination: comparison of effector mechanisms initiated by protein versus DNA vaccination. *J. Immunol.* 166, 3440-3450.

Additional publications:

Tagungsbeiträge

Bauer, H.; Zur Lage, S.; Darji, A.; Lohoff, M. and Weiss, S.: Attenuated *Salmonella* transformed with an eukaryotic vector encoding *Leishmania*-activated C kinase (LACK) as oral vaccine. (Poster and oral presentation) 30th Annual Meeting of the German Society of Immunology, Hannover (1999)

Becker, C.; Bauer, H. and Weiss, S.: Enhancement of tumorspecific CD8 T cells by retroviral transduction with IFN gamma. (Poster) 34th Annual Meeting of the German Society of Immunology, Berlin (2003)

Acknowledgement

Die vorliegende Arbeit wurde an der Gesellschaft für Biotechnologische Forschung mbH (GBF), Braunschweig, in der Arbeitsgruppe Molekulare Immunologie, angefertigt.

Ich danke dem Mentor dieser Arbeit, Herrn Prof. Dr. J. Wehland, für die Betreuung des Promotionsverfahrens, ebenso wie Herrn Prof. Dr. S. Dübel für die Übernahme des Koreferats.

Mein besonderer Dank gilt Herrn Dr. S. Weiß, in dessen Arbeitsgruppe diese Arbeit entstand, für die Betreuung, sein stetes Interesse und seine Diskussionsbereitschaft.

Allen damaligen und derzeitigen Mitarbeitern der Arbeitsgruppe Molekulare Immunologie möchte ich für ihre ständige Hilfs- und Diskussionsbereitschaft, sowie die äußerst angenehme Arbeitsatmosphäre danken. Mein besonderer Dank gilt Susi, Regina und Hanne für ihre tatkräftige Unterstützung im Labor.

Ich danke besonders Herrn Dr. A. Darji und Herrn Prof. Dr. T. Chakraborty von der Universität Gießen für die Ermöglichung der Durchführung und Analyse von Infektionsversuchen.

Petra Beyer danke ich stellvertretend für das gesamte Team des Tierhauses für die gute Zusammenarbeit und die Pflege meiner Mäuse.

All meinen Freunden, Verwandten und Eltern danke ich für die stetige Motivation und Unterstützung während Höhen und vor allem Tiefen in meiner Zeit als Doktorand.

Ein ganz besonderer Dank gilt Michael, der mir immer bei allen Problemen zur Seite stand, mich besonders emotional unterstützt und aufgemuntert hat. Mein größter Dank geht an meinen Sohn Moritz, der durch seine fröhliche Art alle Probleme in den Hintergrund treten ließ und dem ich diese Arbeit widmen möchte.